STUDIES ON A CELL DIVISION CYCLE (cdc) MUTANT OF THE YEAST *Saccharomyces cerevisiae*, DEFECTIVE IN THE INITIATION OF DNA SYNTHESIS.

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ABSTRACT

These studies were undertaken in an attempt to determine the nature of the defect in a temperature-sensitive yeast cell division cycle (cdc) mutant, cdc7.4, which defines an essential step immediately prior to DNA synthesis. The *in vivo* characterisation of this mutant was extended. A yeast mating pheromone, α-factor, was purified and used for cell synchronization purposes. Functional CDC 7 gene product was found to be necessary for nuclear and 2μm plasmid DNA replication, but not for mitochondrial DNA replication, which was unaffected by α-factor. RNA, protein, phospholipid and phosphoprotein syntheses were shown to be unaffected by the mutation.

The *in vivo* characterisation work suggested that the cdc7.4 mutation was due to a temperature-sensitive protein. One- and two-dimensional polyacrylamide gel electrophoresis were used to detect mutationally altered proteins. An abnormal protein present in the original cdc7.4 strain was shown to be unrelated to the lesion in DNA synthesis by a genetic analysis. A second temperature-sensitive defect was also discovered in this strain. An extensively characterised nuclear fraction was analysed on SDS-polyacrylamide gels, and another abnormal band pattern was observed.

Various attempts were made to construct *in vitro* DNA synthesising systems. Controlled lysis of spheroplasts and the fusion of protein-filled liposomes with cdc7.4 spheroplasts were both nominally successful, but were considered unpractical. Reconstitution of a system from purified components was undertaken.

A hybrid plasmid (pJDB219) containing yeast 2 μm DNA sequences was used as the substrate. Several proteins implicated in DNA replication in other systems were partially purified, including DNA polymerases, RNA polymerases, single-stranded DNA binding protein and DNA topoisomerase. These proteins, and partially fractionated endonuclease activity, were shown not to be thermosensitive.

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ABBREVIATIONS

cdc	cell division cycle (mutant function).
CDC	cell division cycle (wild-type function).
ts	temperature-sensitive.
CsCl	caesium chloride.
dATP, dGTP, dCTP, dTTP.	deoxyadenosine, deoxyguanosine, deoxycytidine, deoxythymidine triphosphates.
dTMP	deoxythymidine monophosphate.
rATP, rGTP, rCTP, rUTP	adenosine, guanosine, cytidine uridine triphosphates.
rUMP	uridine monophosphate.
dNTPs	deoxyribonucleoside triphosphates.
rNTPs	ribonucleoside triphosphates.
ssDNA	single-stranded DNA.
dsDNA	double-stranded DNA.
mit.DNA	mitochondrial DNA.
nuc.DNA	nuclear DNA.
β-met	β-mercaptoethanol.
DTT	dithiothreitol.
cAMP	$2^1(3^1)$ adenosine monophosphate.
BSA	bovine serum albumin.
ASO_4	ammonium sulphate.
TCA	trichloroacetic acid.
AAc	acetic acid.
DEAE-	Diethylaminoethyl-
BND	benzoylated, naphthoylated DEAE-
PMSF	phenylmethylsulphonylfluoride.
DMSO	dimethylsulphoxide.
NAD(H)	nicotmamide adenine dinucleotide(H).
SAM	S-adenosyl methionine.

ABBREVIATIONS continued

MA	mevalonic acid.
APT	aminopterin.
SAA	sulphanilamide.
SDS	sodium dodecylsulphate.
PAGE	polyacrylamide gel electrophoresis.
EDTA	ethylene diamine tetraacetic acid.
RNAase	ribonuclease.
DNAase	deoxyribonuclease.
SSC	standard saline citrate (0.15M NaCl, 0.015M Na citrate).
PEG	polyethyleneglycol.
ρ^{o}	no detectable mitochondrial DNA.
ρ^{+}	respiratorily competent.
TEMED	N,N,N',N'-tetramethylenediamine
EGTA	1,2-Di(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid
glusulase	β-glucuronidase
Rif	rifampicin
Stl	streptolydigin
Nal	nalidixic acid
CHM	cycloheximide

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To my parents....

CHAPTER ONE

INTRODUCTION

1.1 An Introduction to the Cell Cycle.

A cell divides by doubling all its structural elements and functional capacities. Studies on the "cell cycle" have attempted to discover how the many complex synthetic and regulatory events involved are integrated into a single pattern. The nucleus, store of the hereditary material, is the key to the process of division. Studies using microscopy, and in the 1950's, radioactive thymidine for the specific labelling of DNA, were able to identify four successive phases of the cycle in growing eukaryotic cells; G1, S, G2, and M (Howard and Pelc,1953). Usually, DNA replication is restricted to S phase; nuclear division takes place in G2, leading to mitosis (M) and cytokinesis. G1 is particularly important, since it contains control steps regulating entry to the cycle. A further state, G0, has been invoked to accommodate quiescent cells. This has become the classical model of the cell cycle. Illuminating exceptions have been discovered, and new ideas have arisen, but for most purposes, this model is still the most instructive.

The bacterial cell cycle was originally thought not to contain an interval corresponding to G1 in eukaryotes, but Helmstetter and Pierucci (1976), showed that slow growing bacteria could display such a phase. It may be dogmatic to think of the cell cycle as a single cycle of dependent events. Hartwell (1974) has shown that the cell cycle of Saccharomyces cerevisiae is in fact composed of two loosely coupled cycles. Recently, Cooper (1979) attempted to explain the presence or absence of the G1 period, by postulating that the preparations for the next S phase are undertaken coincidentally with the start of the current S phase, such that a particular cell will have a discrete "G1" only when the preparations take longer than the collective time for S and G2. It is not the only possible unifying explanation.

At the moment, such models are speculative. This thesis is

concerned with the period of preparation for S phase, specifically those events immediately preceding DNA replication. It may indeed be of service that eukaryotes display an obvious G1 phase, that these preparations may be observed unambiguously. For this reason, sections 1.2 and 1.4 are devoted entirely to eukaryotic cells.

1.2 The Importance of G1.

Attempts to measure the length of the phases of the eukaryotic cell cycle produced a mass of data, summarised by the observation that different cell types have completely different cell cycles (Prescott, 1976). Moreover, the cell cycle times of similar cells within a population are not constant. Total and prolonged synchrony of a population of cells is impossible to attain due to these differences.

1.2.1 The variable length of G1.

Numerous studies have implicated G1 as the most variable phase of the cell cycle. Using time-lapse photography, Petersen et al (1968) studied Chinese hamster ovary (CHO) cells, and showed that the cell cycle time for individual cells could be anything between 10 to 20 hours. However, the length of S+G2+M occupied from 9 to 11 hours, leaving G1 responsible for a 1 to 10 hour variance. G1 variability is not heritable, since the average cell cycle time remains the same throughout many subcultivations. Thus G1, or some step within it, would appear to be the rate determining phase of the cell cycle.

1.2.2 The Control of Cell Reproduction in G1.

Cessation of cell reproduction normally results in arrest of the cell in G1. This can be seen in the transition from exponential growth to stationary phase, when the average length of G1 increases until cells finally become blocked in G1. Van't Hof (1970) induced

stationary phase in cell populations of pea root tips in culture by carbohydrate starvation, and found that the initiation of DNA synthesis was increasingly delayed, but once commenced, the transit times for S+G2 were not affected.

Pardee (1974) showed that in Baby Hamster Kidney (BHK) cells, a diversity of suboptimal nutritional conditions including deprivation of isoleucine, glutamine, serum, phosphate, elevated cAMP levels as well as inhibition by cytochalasin B (affecting cell separation), resulted in the cells arresting at the same point in G1, which was termed the R (restriction) point. This was shown by measuring the time taken for arrested cells to commence DNA synthesis upon restoration of complete medium, which was found to be constant at 8 hours, irrespective of the block or time spent at the block. This was also the case for contact-inhibited Nil 8 cells.

The phenomenon of G1 arrest extends also to differentiated cells. Cells that cease to reproduce, either reversibly (e.g.lymphocytes) or irreversibly (e.g. neurones), usually contain the G1 amount of DNA. Also, in regenerating tissues, the rate of reproduction is governed by the length of time cells spend in G1 (Cameron and Greulich, 1963). In general, the S, G2 and M phases may change in duration, particularly during the slow down of the rapid cell reproduction in embryogenesis (Hoshino et al, 1973), but the greatest variable is the length of retention in G1.

Yeast, a simple eukaryote, shows G1 control most markedly. In the budding yeast, Saccharomyces cerevisiae, haploid cells are one of two mutually exclusive mating types, either a or α. Strains of α-mating type constitutively produce a peptide mating factor (α-factor), which arrests a-type cells in G1 (Bucking-Throm et al, 1973). There is evidence that a-type cells produce a corresponding factor which arrests α-type cells (Betz et al,1977). This arrest in G1 is a prerequisite

for cell fusion and zygote formation (Hartwell,1974). Ultrastructural studies by Byers and Goetsch (1973) confirm this, by showing that mating cells contain a single spindle plaque (1.6), indicative of early G1 synchronization.

Hartwell (1967), produced a set of 148 temperature sensitive cell division cycle (cdc) mutants in 32 complementation groups, defining genes required for progress through the cell cycle in S.cerevisiae. Three of these mutants, cdc4, cdc7, and cdc28 were defined as being blocked in the initiation of DNA replication (Hartwell, 1973). Hereford and Hartwell (1974) were able to place these genes in a temporal sequence for the action of their products relative to the α-factor mediated step. They concluded that the sequence was :

$$\alpha/\text{CDC28} \rightarrow \text{CDC4} \rightarrow \text{CDC7} \rightarrow \text{initiation of DNA synthesis.}$$

The action of α-factor and the cdc28 block could not be temporally separated. Cells arrested at either block were single, unbudded and mononucleate, possessing only one spindle plaque (1.6). The two blocks were said to mediate an early event of the cell cycle, called "Start" (Hartwell et al, 1974). Passing this point, the cell becomes committed to division, to the exclusion of other processes such as mating, until "Start" is regained at the completion of the cycle (Hartwell, 1978).

Nutrient deprivation in yeast, (i.e. carbon, glucose, glycerol, acetate, lactate, ammonia, sulphate, phosphate, biotin or potassium) is known to result in accumulation of cells in G1. Arrested cells were shown to accumulate at, or immediately prior to "Start" (Hartwell, 1974). Wolfner et al,(1975), studied the effect of starvation for arginine, histidine, lysine and tryptophan upon their biosynthetic enzymes. Normal cells derepress these enzymes, but Wolfner et al discovered two sorts of mutants which had lost this control. One type, aas, is unable to derepress, whilst another type, tra, is permanently derepressed for these enzymes. The tra mutants were found to be coincidentally temperature-sensitive for growth, arresting early in the cell cycle.

Moreover, when the tra dependent step was sequenced in relation to the α-factor and cdc4 blocks, by experiments analogous to those of Hereford and Hartwell (1974), it was found to be indistinguishable from the α-factor sensitive step, i.e. "Start".

Sulphate deprivation of S.cerevisiae was studied further by Unger and Hartwell (1974). They used three conditions (sulphate starvation of a prototroph; methionine starvation of an auxotroph; and shift of a methionyl-tRNA synthetase mutant to the restrictive temperature), to define different steps along the pathway for sulphate assimilation. They observed that the imposition of any of these three conditions lead to G1 arrest. Postulating that an intermediate along the pathway was acting as a signal to notify the cell of impending starvation, they concluded that the signal was generated at or after the methionyl-tRNA synthetase step. The signal could be the charged methionyl-tRNA, or a protein required for completion of "Start". At the moment a mechanism cannot be specified, but this work suggests that nutrient "stock-taking" controls regulating entry to the cell cycle may be mediated by a signal at the level of protein biosynthesis.

Taken together, this work on several different systems suggests a ubiquitous control point in G1 which regulates entry into the cell division cycle, and that oncethis point has been passed, the cell is committed to divide.

1.2.3 Is G1 essential?

Certain eukaryotes lack a defined G1 period. Amongst these are Physarum, Amoeba proteus, the micronucleus of Tetrahymena, and rapidly proliferating cells within multicellular organisms (Prescott, 1976). In the case of Amoeba, cessation of cell reproduction by nutrient starvation leads to arrest in G2. Restoration of nutrient supply leads, however, not to mitosis, but to the initiation of a new S phase. This is clearly a more complex situation than those described in 1.2.2, but

it implies that the events leading up to DNA synthesis can in this case be found in G2. Thus, G1 is not a universal attribute of cells, but its presence is genetically and developmentally determined. In such G1-less cases, normal G1 functions can be found in another part of the cell cycle (Prescott, 1976). These systems support the cell cycle model of Cooper (1979), mentioned earlier (1.1).

1.3 Ideas on the Control of Cell Proliferation.

1.3.1 The Transition Probability Model

The variability of cell cycle times described in 1.2.1, particularly the observed variability of G1 in populations of cells, lead Smith and Martin (1973) to propose a modified view of the cell cycle. In this model, G1, S, G2 and M, as definable states, are dispensed with, and replaced by two conditions: an A state (indeterminate), and a B phase (determinate). After mitosis, a cell is said to enter A state, in which its activity is not directed towards replication. It may remain in A state for any length of time ranging from very short periods for rapidly proliferating cells, to greater than 70 years for a quiescent cell. Whilst in this state, there is a constant probability (the transition probability, P), that the cell will enter B phase, whereupon it is committed to division. Transition is random, but influenced by cell type and environmental factors. The relationship of this model to the classical view is shown in Fig.1.

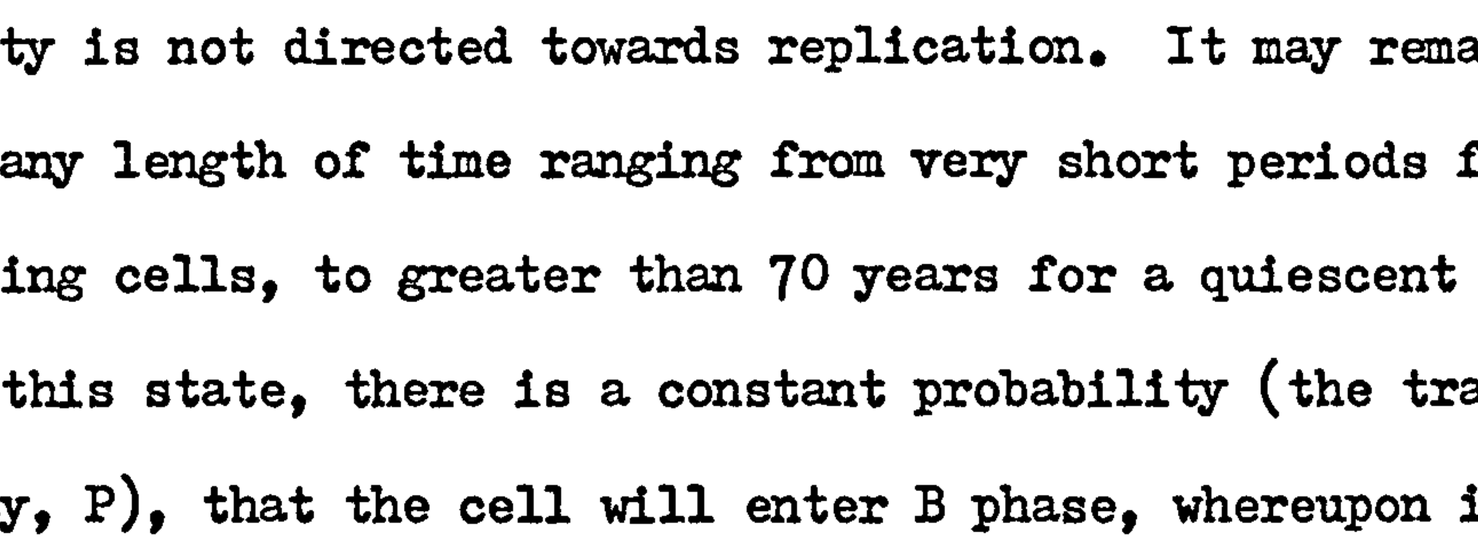

Fig.1. Relationship of the Transition Probability model to the Classical model of the Cell Cycle. (from Smith and Martin, 1973).

Theoretical considerations suggest that the classical model predicts a normal distribution of intermitotic times, in which the probability of division increases with cell age. The Transition Probability model predicts strictly exponential kinetics for cell division (Smith and Martin, 1973). The authors studied various cell types growing in culture by time lapse photography, and produced "α-curves", relating the proportion of cells undivided to time. These curves are exponential after an initial curvature. Shields (1978) showed that a better analysis came from measuring the differences in intermitotic times of sister cells, and plotting these against time (β-curves). These curves are completely exponential, and therefore fit the transition probability model. The initial curvature seen on α-curves, which is not found on the straight line β-curves, is therefore attributable to differences in the length of the determinate B phase in non-sibling cells.

Work with several systems has produced evidence in favour of this model. Brooks (1975) studied serum-stimulated cell division in serum-deprived fibroblasts (1.2.2), and concluded that the cells entered S phase with first order kinetics, the rate constant depending on the concentration of serum used. Suboptimal doses of serum did not commit a subfraction of cells to enter DNA synthesis, but committed the whole population to enter at a slower rate.

Shilo et al (1976) reported that S.cerevisiae showed first order kinetics in cell cycle initiation by using cells released from the α-block and the cdc25 block (a Hartwell mutant, said to arrest at "Start"). By measuring the percentage of unbudded cells remaining after release, they concluded that traverse of "Start" was probabilistic. Criticism of this paper ensued (Nurse and Fantes, 1977), with the suggestion that the kinetics were an artefact of recovery from the imposed block, and that cdc25 was in fact defective in macromolecular synthesis, such that its arrest at "Start" was a secondary pleiotropic

effect (Johnston et al, 1977). Shilo et al, (1977) countered that other genuine "Start" blocks, such as cdc28 and tra3 (Wolfner et al, 1975), produced the same kinetics.

Thus it appears that in yeast there is evidence for the transition probability model. It is tempting to support the elegant transition probability model, since it is capable of interpreting the behaviour of any cell type, depending on the definition of A-state and B-phase. Shields (1976) and Brooks (1976) argue that serum stimulation of fibroblasts does not trigger a sequence of events leading to DNA synthesis, but that it stimulates the formation of a "probability generating function (pgf)", which triggers entry to B-phase. A sequence of events is envisaged in the formation of pgf, but this is said to be in A-state. However, this interpretation needs to be questioned following the work of Pledger et al, (1978). Using density inhibited BALB/c-3T3 cells, they were able to detect growth arrest points in the plasma dependent traverse of G0/G1, i.e. within A-state as defined by Brooks (1976). Earlier experiments, (Pledger et al, 1977) had shown that there are two sets of growth factors in serum, both of which are required for serum-stimulated entry into S phase. One set is termed platelet derived growth factor (PDGF), present in boiled extracts of human platelets, and the other set is present in platelet poor plasma (PPP). Treatment of blocked BALB/c-3T3 cells with PDGF caused them to become "competent". Addition of PPP to competent cells allowed them to enter S phase after a lag of 12 hours. Pretreatment of blocked cells with PPP could not shorten this lag, suggesting that PDGF was necessary for the completion of an initial step. By exposing competent cells to PPP for varying lengths of time, and then removing the plasma stimulus, growth arrest points were detected. Exposure for 10 hours detected an arrest point (V point) 6 hours before DNA synthesis, whilst exposure for 12-15 hours showed an arrest point (W) just prior to DNA synthesis. Exit of cells from the W point was shown

to be a direct function of PPP concentration, and to follow first order kinetics (c.f. Brooks, 1976). Interestingly, commitment to DNA synthesis from W was sensitive to cycloheximide but was insensitive to hydroxyurea, suggesting adequate pools of dNTP's were available, but that there was still a requirement for protein synthesis. The results are summarised in Fig.2.

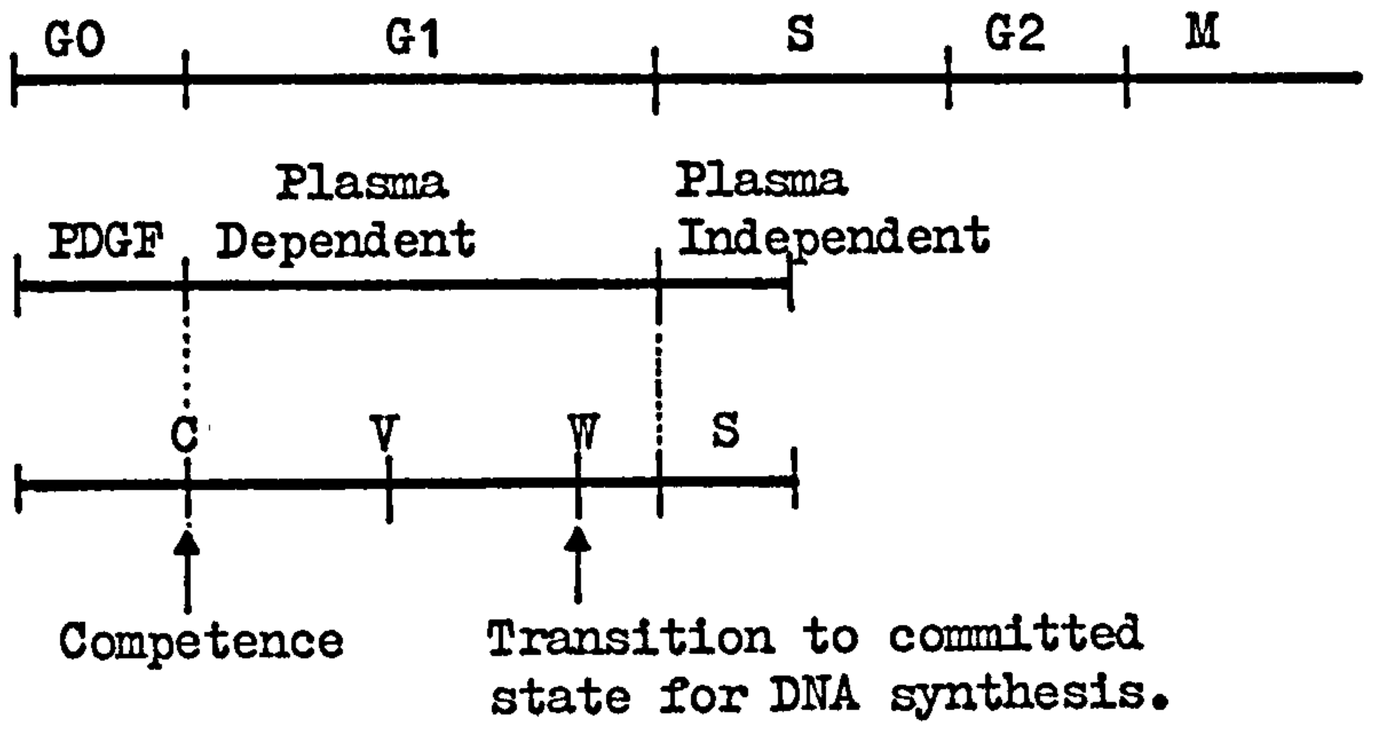

Fig.2. Sequential steps before a transition point in BALB/c-3T3 cells (from Pledger et al, 1978).

These experiments confirm a transition probability step as described by Smith and Martin (1973). However, the transition point was shown to be preceded by an orderly sequence of events, that cannot be attributed to A-state quiescence. In the light of this work, Brooks et al, (1980), have reinterpreted data on the stimulation of quiescent cells by growth factors, and have proposed a unifying model invoking two random transition events per cycle. An indeterminate state, Q, in which serum deprived cells arrest, precedes the familiar indeterminate A-state, the two separated by a lengthy determinate phase (L). The novel feature of this model is the alleged entry into Q state upon leaving A-state, i.e. upon entering S phase, reminiscent of the Cooper (1979) model. The model also explains non-sibling differences which distinguish α and β curves.

1.3.2 The Cell Size Theory.

The idea that cell growth is in some way connected with cell

division has been current for the whole of this century. The reason lies in the observation that particular cells possess a characteristic size, which is rapidly regained upon removal of an insult which perturbs the system. Growth was defined by Swann (1957) as - "a not too precise shorthand for those processes not connected with division which provide the bulk of the new cytoplasm". Mitchison (1971) gave structure to these ideas by postulating the cell cycle as two loosely coupled cycles, the "growth cycle", and the "DNA-division (DD) cycle". In eukaryotes, the latter would be composed of centriole formation, S phase, nuclear division, cytokinesis and cell wall separation, and the growth cycle is said to be the synthesis of most other macromolecules and cell wall structures. Control mechanisms coupling the two cycles have been discussed by Johnston et al, (1977), with reference to S.cerevisiae and the cdc mutants. They argue that the formation of small cells could be avoided if the completion of some event in the DD cycle was dependent on growth beyond a minimum size. Alternatively, a step in the DD cycle could be required for growth beyond a maximum size, thus preventing cells becoming too large. In the first case, cell growth would be rate limiting for cell proliferation, and in the second case, growth would be limited by progress through the DD cycle. Johnston et al, (1977) used the cdc mutants as blocks on the DD cycle, and starvation as a block on the growth cycle, and asked whether the imposition of a block in one cycle affected progress through the other. From measurements of cell weight and volume, extensive growth following blockage of the DD cycle was found. Also, cells were able to complete DD cycles already underway when deprived of nutrients, before arresting in G1 (1.2.2.). By monitoring bud emergence in starved cultures upon nutrient restoration, it was concluded that growth to a minimum size was required before a cell entered the DD cycle. Moreover, in a complex experiment using cdc4, cdc7 and cdc28 strains, they were able to test for the execution of these steps at various times after release,

simultaneously measuring cell size. They concluded that the attainment of a critical size was required before the execution of the cdc steps.

Corroborative evidence came from the studies of Nurse (1975) on the fission yeast Schizosaccharomyces pombe. Nitrosoguanidine mutagenesis produced a temperature-sensitive mutant, subsequently called weel-50 (Nurse and Thuriaux, 1977), which was found to grow to half normal size at the restrictive temperature (Nurse, 1975). This reduction was accompanied by a corresponding reduction in cellular protein and RNA at the time of division. The cell cycle of this mutant appears to be altered, with DNA replication occuring slightly later than in the wild type parent. The defect is due to a single nuclear gene. Wild type cells, grown in conditions which produce small cells (nitrogen starvation), initiate DNA synthesis at cells of similar size to the weel-50 mutant (Nurse and Thuriaux, 1977). Nurse concludes that there are in fact two size controls operative in the cell, one acting over DNA replication, and a second over nuclear division. The weel-50 mutant is said to be defective in this second control, resulting in a requirement for growth to a critical size before entering S phase. In normal cells, the control at this point is argued to be inoperative, since they exceed the critical size at division. Mitchison (1977), argues that these results remove the requirement for a "timer" control on the cell cycle.

However, several criticisms can be made of the original work. The cell cycle time of the weel-50 mutant is exactly the same as that of the wild type parent (Nurse, 1975). For cells to grow to half normal size, containing half quantities of RNA and protein at the restrictive temperature suggests a primary lesion at the level of general RNA or protein biosynthesis, with cell cycle transit time in the control of a "timer" mechanism. If a cell size control operates, assuming there is no reduction in the rates of RNA and protein synthesis, in the weel-50 mutant, the transit time should be reduced. Similarly,

criticism can be made of the work of Johnston et al, (1977) on S.cerevisiae. Since the cdc mutants used all display the characteristics of first cycle arrest (Hartwell, 1974), and de novo protein synthesis is required for the traverse from "Start" to the initiation of DNA synthesis (Hereford and Hartwell, 1973), clearly some, or all, of the proteins required for this transit are not present in G1 arrested cells. Thus, any lag phase observed upon exit from "Start" could correspond to the synthesis of essential proteins, the correspondence with cell size being fortuitous.

The most convincing evidence favouring control through cell size came from studies on bacteria into the nature of the regulation of DNA synthesis. Early work by Schaecter et al, (1958), demonstrated that average cell mass increases continuously with growth rate in Salmonella typhimurium. Rapidly growing bacteria were shown to contain multiple replication forks by Yoshikawa et al, (1964). Maalφe and Kjeldgaard (1966) concluded that multiple fork replication occurs when the rate of DNA synthesis using a single replication point becomes limiting. Cooper and Helmstetter (1968), suggested that these effects could be understood by defining two constant time intervals: C, the time taken for a replication point to proceed from origin to terminus, and D, the time between the end of a round of chromosome replication and division. To fit observed data, C and D were allotted the values 40 minutes and 20 minutes respectively. A set of theoretical chromosome configurations was generated from these constants, from which could be predicted the relative rate of DNA synthesis in cells growing at different rates. When measured, these fitted the theoretical values (Cooper and Helmstetter, 1968).

Donachie (1968) combined the observations of Schaecter et al, (1958), with the Cooper-Helmstetter model, to show that the initiation of successive rounds of chromosome replication was strikingly correlated with the attainment of integral values of cell mass. Jacob et al, (1963),

had already suggested the "replicon" model for DNA synthesis regulation, by analogy with transcriptional (repressor/operator) control. They postulated that each unit of replication, or "replicon" carries information for an initiator molecule which governs the replication of that same replicon. The system envisaged positive control by the initiator. Donachie (1968) used this idea to suggest that a cellular initiator substance was produced at a rate proportional to overall increase in mass. A certain critical level of initiator, corresponding to a critical cell size, has to be reached before the initiation of DNA synthesis can be achieved, whereupon the initiator molecules are consumed.

A negative model of the initiation of DNA synthesis arose at a similar time. Pritchard et al (1969) suggested, on the same theoretical grounds, the existence of a constitutively produced initiator protein (I), and an inhibitor (H) protein transribed at the time of replication. The two proteins are said to interact, such that growth beyond a critical level is required to overcome inhibition, which leads to initiation, followed by the further synthesis of inhibitor which blocks further initiation. The model neatly explains why initiation is a one-off event, not repeated until extra growth allows. Again the postulate of a critical cell size is a central feature. A further sophistication of this model was proposed by Sompayrac and Maaloe, (1973). Initiator protein (p_2) is under the control of an autorepressor, (p_1), such that repression varies with growth rate (Fig.3). The model postulates that initiation takes place when at each initiation site a structure is built containing a fixed number of initiator molecules, which are consumed in the act of initiation.

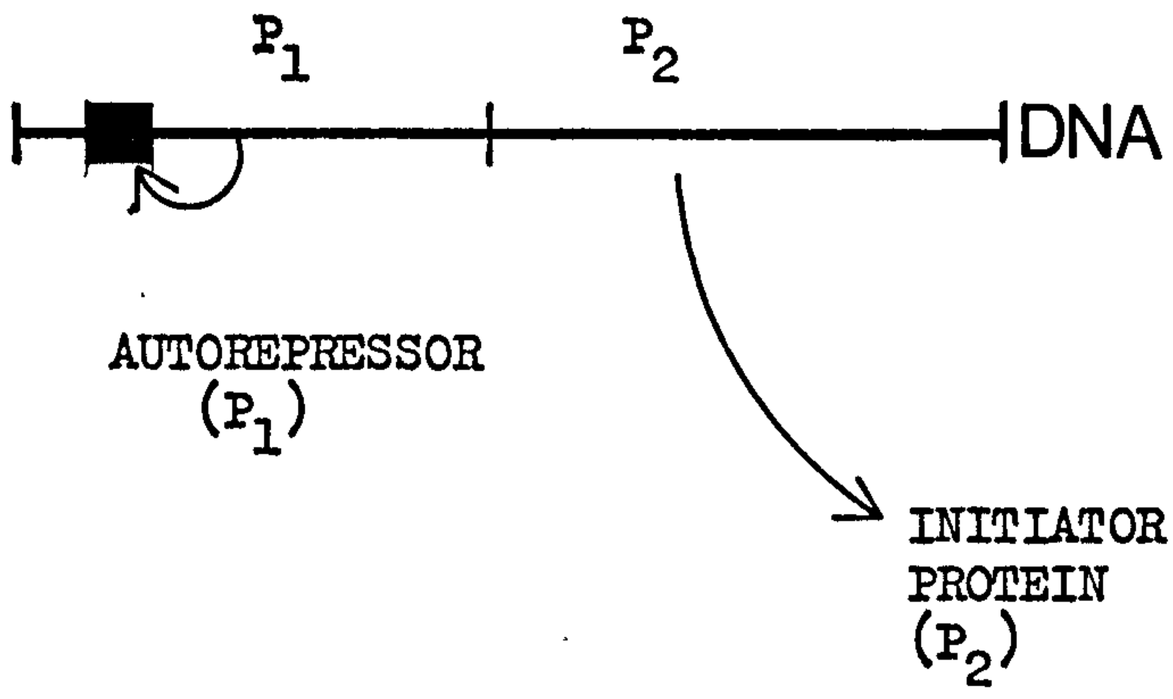

Fig.3. The Autorepressor Model for Control of DNA Replication (from Sompayrac and Maaløe, 1973).

Pritchard (1978) has shown that the inhibitor dilution model can be used to explain the phenomena of stringent and relaxed plasmid replication, plasmid copy number control, plasmid incompatibility and integrative suppression (1.5.3.1). The ageing replicon model cannot satisfactorily explain these effects. However, the molecular support for any of these theories is yet to be presented. Perhaps the discovery of a 100 nucleotide transcript from colE1 (Conrad and Campbell, 1979) which is implicated in the initiation of DNA synthesis (1.5.4.3) and is also involved in the control of plasmid copy number, will provide the necessary molecular foothold.

1.3.3 Summary.

Sections 1.2 and 1.3 have attempted to establish the idea that major control points for cell proliferation exist prior to DNA synthesis. Cell population kinetics have demonstrated the existence of restriction points in the prereplicative phase of the cell cycle. Growth and division are intimately linked, but it is not true to say that cell size controls cell proliferation. The attainment of a particular cell size is due to the sum of the metabolic and biosynthetic processes within the cell. In embryonic tissues, rapid cell division

is not accompanied by corresponding increases in overall mass (Graham, 1973). Alfert (1950) showed that the nuclear volume halved with each division in early cleavage embryos. Thus the critical size phenomenon is related to the particular stage of differentiation and development of a cell.

These ideas have not yet elucidated the means by which proliferation is controlled, but they are important nevertheless, since they question the blind adoption of any rigid cell cycle model, with its attendant misconceptions.

1.4. Entry into S phase.

1.4.1. Nuclear-cytoplasmic interactions in DNA synthesis.

Numerous lines of research have indicated that cytoplasmic factors are involved in the initiation of S phase. Experiments have involved observations on multinucleate cells sharing a common cytoplasm, artificially induced fusion of cells (heterokaryons), and microdissection and transplantation of nuclei.

The thousands of nuclei in the syncytium of Physarum polycephalum, initiate DNA synthesis synchronously (Nygaard et al, 1960), indicating common control mediated through the cytoplasm. Also, in organisms which are normally mononucleate, binucleate cells sometimes arise, or can be induced. The cilian protozoan Euplotes can spontaneously generate binucleate cells, in which case both macronuclei enter S phase at the same time, (Kimball & Prescott, 1962). Gonzalez-Fernandez et al (1971) showed that in caffeine-induced multinucleate onion root cells, all nuclei in the same cell commenced DNA synthesis synchronously.

The heterokaryon studies of Johnson & Harris (1969. a,b,c) produced some important findings. Chick erythrocytes, normally arrested permanently in G1, were induced to synthesise DNA when fused with HeLa cells (Johnson & Harris, 1969 b). Both nuclei entered S phase synchronously, indicating that the factor(s) present in the HeLa

cell cytoplasm which cause DNA synthesis initiation were not species specific. Similar studies using HeLa-mouse, and HeLa-hamster heterokaryons, where the mouse and hamster parents are characterised by shorter G1 phases than the HeLa cells, showed that in both cases, the nuclei in the heterokaryons synthesised DNA with the shorter G1 phase (Graves, 1972). However, each nucleus was found to spend its characteristic time in S phase, indicating that the programme of DNA replication is an intrinsic feature of the nucleus.

Once S phase has been started, it appears to be completed, irrespective of the cytoplasmic background. Ord (1969, 1973), transplanted S phase nuclei of Amoeba proteus into G2 phase cells, or even early S nuclei into late S cells, and showed that the donated nucleus always completed its own programme. The lack of a defined G1 phase in this organism (1.2.3), may account for differing observations made by other workers (Prescott, 1976). Certainly, the results of Ord (1969) appear to concur with those of Rao & Johnson (1970) using virus-induced fusion of HeLa cells at different times in their cell cycles. They again showed that S phase cells completed their DNA synthesis when fused with G2 cells, and pointed out that the G2 cells were not induced to enter a new S phase. However the G1 phase could be shortened by fusing cells in this phase with increasing numbers of S phase cells.

All of the work described here favours the idea of a cytoplasmic inducer of DNA synthesis present only during S phase, which acts on the nucleus. G2 cells do not respond to this inducer, but likewise do not produce a cytoplasmic inhibitor, since S phase can be completed in G2 cytoplasm.

Of relevance in this field is the work of Jazwinski & Edelman (1976), who showed that extracts prepared from the yeast cell division cycle mutants cdc 28, cdc 4, cdc7, could stimulate DNA synthesis in isolated nuclei of the frog, Xenopus laevis, when the yeast cultures had been grown at the permissive temperature (23°C), but not if grown at the

restrictive temperature (36°- 38°C) for one generation. The wild type parent A364A could be grown at either temperature to produce active extracts. Whilst not bringing an understanding of the underlying molecular biology any closer, this work shows the remarkable lack of species specificity (i.e. the highly conserved nature) of the factor(s) bringing about the transit from G1 to S phase.

1.4.2. A determined sequence of events as a prelude to DNA synthesis.

Reference has already been made to two systems which provide evidence for the existance of a determined sequence of events in the prereplicative phase of the cell cycle (1.2 and 1.3). For S.cerevisiae, Hereford & Hartwell (1974) were able to place the cdc 28, cdc 4 and cdc 7 genes in a temporal order, with respect to the action of their gene products, using reciprocal shift experiments, (Jarvick & Bolstein, 1973). The temperature and α-factor blocks were the alternative restrictive conditions. The construction of double mutants enabled verification of the dependent sequence as (α/28>4>7>initiation of DNA synthesis). In the same experiments, they were able to demonstrate a requirement for essential protein synthesis between the cdc 4 and cdc 7 stages. When cycloheximide was added to cultures synchronised at the cdc block, and the culture shifted to the permissive temperature, a burst of DNA synthesis was seen in cdc 7 cultures, but not in cdc 4 or cdc 28 cultures. Evidence was presented later (Edwards, et al, 1978), that there may be a further protein synthesis requirement at the start of the cell cycle.

The studies of Pledger et al (1978) described a sequential programme in BALB / c-3T3 cells leading from a quiescent state to a transition point, W, immediately before DNA synthesis (1.3.1.). Knowledge has been further advanced by the isolation of numerous temperature sensitive cell division cycle mutants in mammalian cells, (review Simchen, 1978). Mutants blocked in G1 are of particular interest.

Amongst these are tsAF8 (Burstin et al, 1974) ts13, ts11 and tsHJ4 (Talavera and Basilico, 1977) all in BHK cells, and K-12 (Roscoe et al, 1973) in Chinese hamster cells. Using one of these, AF8, Burstin et al, (1974) were able to distinguish cell cycle arrest due to serum starvation from arrest due to isoleucine deprivation. In reciprocal shift type experiments, they looked for DNA synthesis at the restrictive temperature (30.5°C), following readdition of serum or isoleucine. After serum replacement, cells remained blocked at 39.5°C, whereas isoleucine replacement resulted in transit of the cells into S phase. This indicated that the AF8 mutation mediated a step between the two blocks. Talavera & Basilico (1977) isolated three temperature-sensitive mutants, (ts13, ts11 and ts HJ4), all of which arrested between the isoleucine deprivation block, and the hydroxyurea block i.e. entry into S phase, (Slater, (1973): Hartwell, (1976). The lesions were tentatively sequenced in this interval, by following the timing of entry into S phase of synchronous cultures, and estimating the time at which the cultures became unresponsive to a shift to the restrictive temperature (the execution point). The tsHJ4 defect was thought to be expressed at the time of entry into S phase, on the basis of experiments on cells synchronised with hydroxyurea at the G1/S boundary, and then released and shifted simultaneously to 39.5°C. Under such conditions, ts11 and ts13 could complete S phase, but tsHJ4 allowed only partial entry.

Ashihara et al, (1978) used five different growth conditions to relate the execution points of the G1 mutants AF8 (BHK cells) and K-12 (Chinese hamster cells) to the entry into S phase. The results convincingly show the K-12 and AF8 steps to lie 1.8 hours and 8.6 hours prior to S phase, respectively. All four mutations (ts11, ts13, K-12, tsHJ4) define different genes (Talavera & Basilico 1977). Thus a possible sequence of events, defined by these genes and metabolic blocks could be proposed (Fig.4). Jonak and Baserga (1979) using AF8 and ts13

cells, found evidence from studies of fusion of cytoplasts with whole cells (both G0) that the information for these two temperature sensitive functions was already present in the cytoplasm of G0 cells before serum stimulation committed them to proliferation. This suggests that control of this part of the cell cycle may be mediated through translation or post-translational modification. Whether this picture can be improved by the discovery of more and better mutants remains to be seen. At the moment, there is no knowledge of the molecular events mediated by these, or any other genes involved in G1 transit.

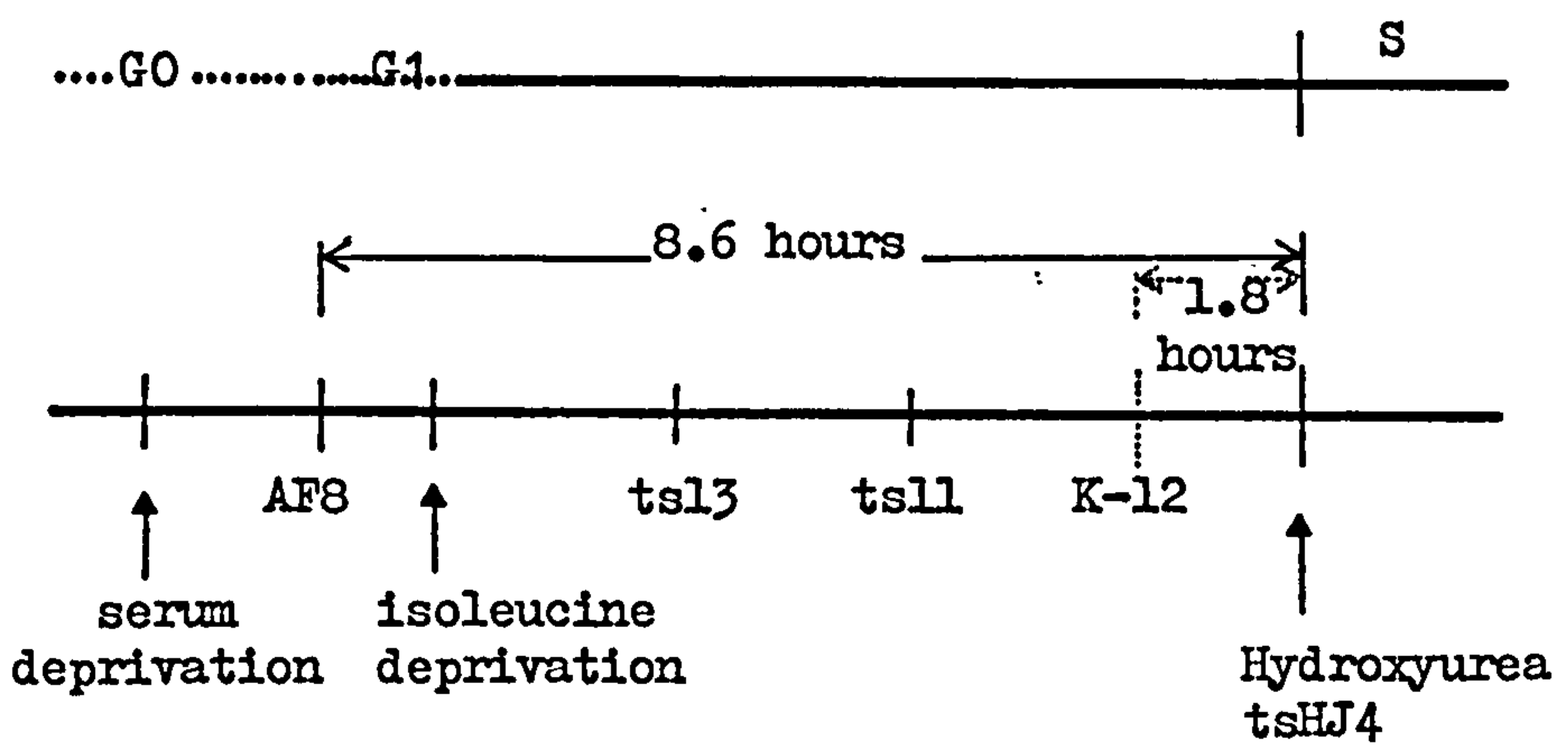

Fig.4. G1 in BHK/Chinese Hamster cells (from Talavera and Basilico, 1977; Ashihara et al, 1978; Burstin et al, 1974).

There appears to be a ubiquitous requirement for protein synthesis to complete G1. Experiments involving cycloheximide inhibition, and amino acid deprivation have shown that continuous protein synthesis is necessary to allow entry into S phase (Brooks, 1977). Analysis of the components bringing about DNA synthesis has been dependent on the development of in vitro DNA synthesising systems. These have yielded considerable information in bacterial and viral studies (review, Wickner, 1978). These will be discussed fully in the next section (1.5).

It is pertinent to point out that other effectors have been implicated in regulating transit through G1, particularly cyclic nucleotides (Clarkson & Baserga, 1974). There is much evidence to

suggest a cell cycle dependence of the appearance of various cAMP dependent protein kinases and cAMP itself. Costa et al (1976) identified two cAMP dependent protein kinase activities in CHO cells, termed Type I and Type II. Total amounts of both enzymes drop in G1, but a rapid increase in Type II enzyme was seen at the G1/S boundary. High levels of intracellular cAMP are known to be associated with arrest of cells in G1 due to serum deprivation (Sheppard & Bannai, 1974). There is also evidence that a rise and fall of intracellular cAMP is required for the onset of DNA replication in lymphocytes (Wang et al, 1978). Tobey et al (1974) presented evidence for cell cycle variability in the synthesis and phosphorylation of histones in CHO cells. Particularly, they suggest that phosphorylation of H1 (or a subpopulation) takes place early in G1. New H1 synthesis occurs immediately prior to DNA synthesis. At present it is difficult to decide on the temporal relevance of these observations, since histone synthesis is variably coupled to DNA synthesis in different eukaryotes (Garcia-Herdugo, et al, 1977). Similarly the observations of Gerner and Humphrey (1973) on the synthesis of a set of non-histone proteins in CHO cells of molecular weight over 45,000 late in G1, and Chang et al (1978) regarding the methylation of 40S ribosomal proteins in HeLa cells in late G1, may be peculiarities of a particular system, unrelated to primary control processes.

1.5. DNA Synthesis.

1.5.1. Introduction.

When Watson and Crick (1953) described the double helical structure of DNA, the means of its faithful replication appeared self evident. Twenty seven years later, it is clear that this process is not a simple enzymatic step. This section deals with current knowledge of DNA synthesis in prokaryotic and eukaryotic systems, with an emphasis on those components implicated in initiation.

DNA polymerases have been isolated from many sources, and studied extensively. (Kornberg, 1980; Holmes and Johnston, 1976). A unifying feature of all DNA polymerases is their inability to initiate synthesis on double stranded or completely single stranded DNA templates. RNA polymerases can initiate synthesis of RNA without a primer, and DNA polymerases can use this primer to synthesise DNA chains. Another common feature is that all DNA polymerases add nucleotides in the 5'→3' direction. Replication of the opposing (lagging) strand is achieved by multiple initiations, producing Okazaki fragments which are subsequently joined (Okazaki et al, 1978). Two roles for priming can thus be recognised; one at the start of replication of the genome, and one as an initiation mechanism of the small fragments made during elongation of the progeny strands.

Much work has been conducted in the last ten years on replication of the various bacteriophages and plasmids resident in E. coli, since these are simple replicons, and lend themselves to analysis much more easily than the complex E. coli chromosome. Replication proteins have been identified in bacteria by the production of temperature sensitive (dna) mutants defective in DNA replication, and by the development of in vitro DNA synthesising systems (Wickner, 1978). Several proteins purified by the latter method have not yet been allocated genetic loci (Wechsler, 1978). Understanding of DNA replication in eukaryotes is comparatively small, due to the lack of suitable mutants and in vitro systems.

Further information has been gained by looking for functions which might be expected to participate in DNA replication. The structure of DNA presents topological problems for its replication, since the strands must be separated. Champoux (1978) has reviewed the work on proteins that affect the conformation of DNA. Four classes can be defined: a. proteins that bind specifically and cooperatively to single stranded DNA, helix-destabilising proteins (Alberts and Sternglanz, 1977), which

are required in all reconstituted systems using single stranded DNA templates. Other proteins in this category include those which melt duplex DNA, e.g. RNA polymerase (Wang et al, 1977). b. enzymes coupling ATP hydrolysis to the unwinding of the two strands, termed unwinding enzymes, or helicases (Kuhn et al, 1979). The E. coli rep protein is an example, which is essential for ØX174RF replication (Scott et al, 1977). c. enzymes which introduce transient nicks into duplex DNA, termed DNA swivelases or nicking-closing enzymes. The ω-protein of E. coli is an example (Wang, 1971), but this does not appear to be involved in replication. d. enzymes which introduce negatively superhelical turns into topologically closed DNA, i.e. DNA gyrase (Gellert et al, 1976a). DNA gyrase is involved in DNA replication (Gellert et al, 1976b); there is also evidence that it is required for specific transcriptional events (Smith et al, 1978).

1.5.2. Functions involved in prokaryotic DNA replication.

Wechsler (1978) has reviewed the genetics of E. coli replication. Thermosensitive or cryosensitive mutants fall into two phenotypic categories, either initiation or elongation defective. Elongation mutants cease DNA replication immediately upon shift to their restrictive temperature, whereas initiation mutants allow completion of those rounds in progress. The antibiotics chloramphenicol and rifampicin have been used to probe the requirements for protein and RNA synthesis respectively. Both affect initiation of new rounds, but rifampicin affects a step after the requirement for protein synthesis is completed (Zyskind et al, 1977). Two more antibiotics, novobiocin and nalidixic acid, cause immediate inhibition of DNA synthesis, and their targets have been identified as the two subunits of DNA gyrase (Gellert et al, 1976b; Sugino et al, 1977). Current knowledge of proteins involved in the initiation and elongation of E. coli DNA replication is summarised in Table I.

TABLE I: E.coli functions required for DNA replication.

LOCUS	CHARACTERISATION
Required for initiation of chromosomal replication	
dnaA	Positively acting regulatory protein (transcription antiterminator ?).
dnaB252	250,000MW active protein, 48,000MW subunit. 10 molecules/cell; ss DNA dependent rNTPase activity. Possible role of mobile promoter used by dnaG in elongation.
dnaC(D)	25,000MW active protein. Interacts physically with dnaB gene product.
dnaI	?
dnaP	?
rpoB	RNA polymerase β subunit. Mutants can suppress dnaA mutation.
Required for elongation of chromosomal replication	
dnaB	see above
dnaC(D)	see above
dnaG	65,000MW active protein. Priming protein which synthesises mixed ribo- and deoxyribo- oligonucleotides, 10 molecules/cell
dnaE	αsubunit of DNA polymerase core enzyme; 10 molecules/cell
dnaZ	=DNA EF II =γsubunit of DNA polymerase holoenzyme. 125,000MW active protein (Wickner, 1976), 52,000MW active protein (Hubscher and Kornberg, 1979) Interacts with DNA EF III
dnaX	DNA EF III = δ subunit of DNA polymerase III holoenzyme. 32,000MW active protein, forms complex with dnaZ.
lig	DNA ligase
polAex	5'→3' exonuclease of DNA polymerase I
cou	component of DNA gyrase sensitive to novobiocin and coumermycin
nal	component of DNA gyrase sensitive to oxolinic acid and nalidixic acid DNA gyrase may be required for initiation to produce correct superhelicity
rep	67,000MW active protein; ss.DNA dependent ATPase. Required for ØX174 RF replication, not essential for E.coli growth. Directional specificity 3'→5' (Yarranton and Gefter,1979). Helicase III is an enzyme functionally similar to rep, but works in 5'→3' direction (Yarranton et al, 1979). Other helicases may also be involved - DNA helicase I and II (Kuhn et al, 1979), explaining lack of dependence on a single enzyme.

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TABLE I: E.coli functions required for DNA replication

LOCUS	CHARACTERISATION
Required for in vitro DNA replication	
?	DNA binding protein (Helix destabliser) 80,000MW active protein, 18,500MW subunit; binds to single stranded DNA.
?	copol III*=DNA EF I = β subunit of DNA polymerase holoenzyme; 40,000MW active protein; involved in elongation of primed single-stranded DNA.
?	Replication factors X,Y,Z = factors i,n',n; required for transfer of dnaB to ϕX174 DNA covered with DBP, in conjunction with dnaC. Factors Y and Z bind to single stranded DNA: Y has ss.DNA dependent ATPase activity.
?	protein u. Involved in ϕX174 $\ominus$ strand synthesis.
Required for termination?	
dnaT	Affects stability and/or termination of replication (Lark and Lark, 1978). Interacts with dnaC gene product.

Notes: dnaJ, dnaK, dnaL, dnaM also define loci involved in replication, but are currently inadequately characterised.
dnaS = dut = sof, dUTPase activity. dnaF is ribonucleotide reductase.

From: Wickner (1978), with additional information.

A number of single stranded bacteriophages have been used as model systems. These include filamentous 'phages M13, fd, f1 and isometric 'phages G3, ØX174, St-1. Their special mode of replication, involving 3 steps (viral SS→RF: parental RF→progeny RF: RF→viral SS;) defines specific problems in priming and elongation. The priming requirements vary between 'phages, from the minimal needs of G4 for host dnaG gene product and helix destablising protein, to the much more complex system of ØX174 which requires many of the products of known genetic loci, and has also defined other factors (Wickner and Hurwitz, 1974, 1.5.4.1).

Other bacteriophages, e.g. λ, T4, T7, depend to varying extents on their own 'phage encoded proteins. T4 does not require any host function, but ten 'phage proteins involved have been isolated, (Liu et al, 1978; Liu et al, 1979).

ColE1 is a colicinogenic plasmid present in E. coli at about 30 copies/cell, and like E. coli chromosome, it is a supercoiled closed circular duplex DNA. In vivo studies have shown a dependence for replication on all the dna gene products with the exception of dnaA (Collins et al, 1975).

These various systems have been extremely useful in studying the problem of DNA replication. A brief summary of the functions involved is presented in Table II (from Wickner, 1978). The following three sections detail the information that they have provided for understanding the initiation of DNA replication.

1.5.3. Initiation of E. coli chromosomes replication.

1.5.3.1. The dnaA mutation.

The initiation of a new round of replication in E. coli is known to require protein synthesis (Lark et al, 1963) and RNA synthesis (Lark, 1972). An initiation mutant of B. subtilis was shown to be blocked at the restrictive temperature after the requirement for protein

TABLE II: Protein requirements of some procaryotic DNA replicating systems.

	E.coli in vivo	fd DNA in vitro	G4DNA in vitro	X174 DNA in vitro	X174 RF 1 DNA in vitro	ColE1 DNA in vitro	λ phage in vitro	T7 phage in vitro	T4 phage in vitro
E.coli functions									
dnaA	+[a]	−[b]	−	−	nt[c]	−	−	−	−
dnaI,dnaP	+	−	−	−	nt	nt	−	−	−
dnaB	+	−	−	+	+	+	+	−	−
dnaC(D)	+	−	−	+	+	+	−	−	−
dnaG	+	−	+	+	+	nt	+	−	−
dnaZ	+	+	+	+	+	+	+	−	−
polC(DNA pol III)	+	+	+	+	+	+	+	−	−
rpoB(RNA pol)	+	+	−	−	−	+	+	−	−
nalA(DNA gyrase)	+	−	−	−	+	+	nt	nt	−
cou(DNA gyrase)	+	−	−	−	+	+	nt	+	−
lig(DNA ligase)	+	−	−	−	nt	+	+	−	−
polA(DNA pol I)	−	−	−	−	−	−	−	−	−
rep	−	−	−	−	+	nt	−	−	−
tsnB,tsnC	−	−	−	−	nt	nt	−	+	−
grpC(dnaJ, dnaK)	+	−	−	−	nt	nt	+	−	−
grpD,grpE	−	−	−	−	nt	nt	+	−	−
DNA binding protein	nt	+	+	+	nt	nt	nt	nt	nt
DNA EF I, protein copol III	nt	+	+	+	nt	nt	nt	nt	nt
DNA EF III	nt	+	+	+	nt	nt	nt	nt	nt
Replication factors X, Y, Z;i, n	nt	−	−	+	nt	nt	nt	nt	nt
Phage functions	−	−	−	−	+	−	+	+	+

[a] Needed for replication.
[b] Not needed for replication.
[c] Not tested.

synthesis, but before a transcriptional step, as shown by its sensitivity to rifampicin and streptolydigin (Laurent, 1973). The sequence of events in E. coli was analysed most thoroughly by Zyskind et al, (1977), who looked at the reinitiation of DNA synthesis in blocked cultures of dnaA and dnaC mutants, when returned to the permissive temperature in the presence of rifampicin, streptolydigin, chloramphenicol, or nalidixic acid. They were able to demonstrate that dnaA acts before or during the synthesis of an origin-RNA; that RNA polymerase synthesised this RNA, and that dnaC gene product was involved in a step after this, immediately before the start of deoxyribonucleotide polymerisation. Zyskind and Smith (1977) studied an unusual dnaB mutation (dnaB252) and were able, by the same crteria, to show that this defect was also expressed before or during the synthesis of an origin-RNA. By the sensitivity of this step to streptolydigin, it was identified as an earlier step than that involving the dnaA gene product. The results of their observations are shown in Fig. 5. Kung and Glaser, (1978) used a double mutant, dnaA (thermosensitive) dnaC (cryosensitive) to confirm that the dnaA gene product acted before that of the dnaC gene.

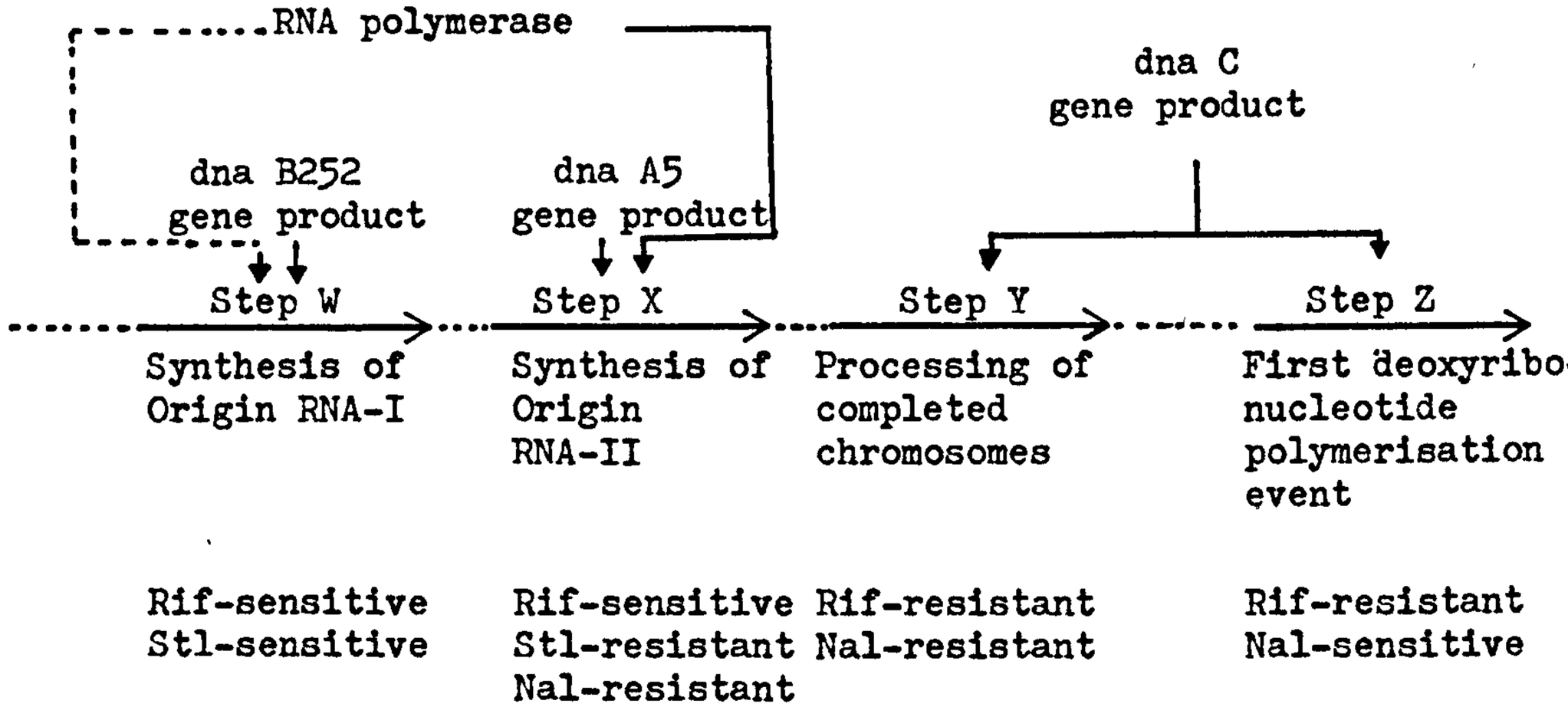

Fig. 5. Order of events during E. coli chromosome initiation. (from Zyskind et al, 1977).

From analysis of the transient stimulation of replication by chloramphenicol in dnaA strains, a negative control of initiation was

proposed in which the dnaA gene product was either the repressor of o-RNA transcription, or was responsible for the removal of the repressor (Messer et al, 1975). Further work gave support for the existence of a rapidly turning-over negatively acting control protein, which was not the dnaA gene product (Tippe-Schindler et al, 1979). By studying the effects of gene dosage of the dnaA gene in various constructed merogenotes, Zahn and Messer (1979) suggested a positive role for the dnaA gene product.

In molecular terms, little is known of the dnaA gene product. Bagdasarian et al (1977) showed that the dnaA phenotype could be suppressed by mutations in the rpoB cistron, coding for β subunit of RNA polymerase in E. coli and S. typhimurium. This is possibly evidence of an interaction between the dnaA gene product and RNA polymerase. Eaton et al, (1979) produced further evidence by showing a temperature dependent release of $\beta\beta'$ subunits of RNA polymerase from folded nucleoids specifically in strains carrying the dnaA mutation. Bagdasarian et al (1978) showed that certain rpoB mutations caused a reduction in thepolarity of hisC amber mutations in the his operon of S. typhimurium. This is circumstantial evidence to support the hypothesis that the rpoB mutations may affect recognition by a transcription antiterminator, and thereby that this may be the role of the dnaA gene product.

The dnaA gene product has never been isolated. None of the studied in vitro systems require a functioning dnaA gene product, nor do bacteriophages or plasmids in vivo (Tomizawa and Seltzer, 1979). The only exception is RF replication of M13 (Mitra and Stallions, 1976) but here again dnaA is not required in vitro. The apparent specific involvement of dnaA at the chromosomal origin is illustrated by the phenomenon of integrative suppression. The integrated R factor R100.1 is typical of many R and F factors in being able to suppress the thermosensitivity of the dnaA lesion (Chandler et al, 1977). Such

suppression is achieved by initiation from the origin of the integrated plasmid at restrictive temperatures.

1.5.3.2. The dnaB mutation.

The dnaB gene product has been purified using a ØX174 *in vitro* system, and shown to be a single stranded DNA dependent NTPase, of native molecular weight 290,000, composed of 6 subunits of 48,000 each (Reha-Krantz and Hurwitz, 1978 a,b). The dnaB252 is an initiation mutant (Zyskind and Smith, 1977) which can be distinguished from other dnaB elongation mutations (dnaBts) by other criteria, i.e. dnaB252 will support λ DNA replication and phage production at the restrictive temperature whereas dnaBts will not (Zyskind and Smith, 1977). Lanka *et al* (1978) have purified dnaB252 gene product and show that this retains ATPase activity. The ATPase activity of the two sorts of dnaB gene product have yet to be directly compared for their temperature sensitivity, but it is suggested that this will be defective in elongation mutants and normal in dnaB252 (Tomizawa and Selzer, 1979).

1.5.3.3. Other genes involved in initiation.

The generality of this title reflects the lack of information on the remaining genes involved in initiation. The role of dnaC has been discussed in 1.5.2.1. The dnaC and dnaB gene products interact physically (Wickner and Hurwitz, 1975). dnaP was isolated on the basis of its resistance to phenethyl alcohol, by Wada and Yura (1974), and may specify a membrane function. dnaJ and dnaK were originally isolated as grpCD mutants unable to support phage λ replication (Saito and Uchida, 1978; Yochem *et al*, 1978.) Little is known of these mutations. dnaG is normally classified as an elongation mutation, but comparative studies of various origin sequences have indicated that dnaG gene product (primase) may be used for priming of *E. coli* chromosomal origin, as will be discussed later (1.5.5).

1.5.4. The initiation of replication in bacteriophage systems.

1.5.4.1. Priming on single stranded circular DNA.

Three priming mechanisms are used by a range of single stranded 'phages. These include priming by RNA polymerase(fd), priming by dnaG gene product (G4, St-1) and priming by dnaG gene product together with other proteins (ØX174).

DNA synthesis on the ⊕ strand of the fd requires RNA polymerase, as shown by its sensitivity to rifampicin (Wickner et al, 1972). Synthesis can be achieved using only RNA polymerase, DNA binding protein (DBP) and DNA elongation components. (Geider and Kornberg, 1974). Exhaustive DNAse digestion of fd DNA complexed with RNA polymerase and DBP yields a protected stretch of 120 nucleotides (Schaller et al, 1976), which contains the ⊖ strand origin (Gray et al, 1978). The sequence can fold into two hairpin loops, but only one of these is essential for DNA replication (Schaller, 1978). Incubation of fd DNA with RNA polymerase, DBP and rNTPS results in the formation of a 30 base transcript (ori-RNA) which was shown to commence at the base of the essential hairpin and continue to the top of the loop (Schaller, 1978).

One step up in complexity is the G4 system in which ⊖ strands can be synthesised by dnaG protein, DBP and elongation components (Rowen and Kornberg, 1978) Primer formation can be shown to be a three step process (Wickner, 1978b). Firstly G4 must be covered by DBP, then dnaG protein binds (1 molecule/G4 circle). The third stage is primer synthesis by dnaG protein, using either dGTP and dTTP or GTP and UTP, which can be stimulated by ADP. Rowen and Kornberg (1978) also showed that the dnaG protein (Primase) can utilise either ribo- or deoxyribonucleoside triphosphates. A unique oligonucleotide of 25-28 residues (Bouché et al, 1978) is synthesised, which extends from the base of a hairpin structure (Godson 1978). The significance of these origin sequences will be discussed later (1.5.6).

The third priming system, and the most complex, is that used in ØX174 ⊖ strand synthesis, requiring at least eleven proteins. The additional factors are the dnaB and dnaC proteins, and factors X,Y and Z (i,n,n), (Wickner and Hurwitz, 1974; Meyer et al, 1978). All of these proteins are required before dnaG synthesises a primer. Firstly, DBP covers the DNA and one or several molecules of Y and Z bind also. Then dnaB protein is transferred to the protein covered DNA in a reaction requiring dnaC, factor X, and ATP, to give a complex containing ØX174DNA⊕, DBP, dnaB, and factors Y and Z (Weiner et al, 1976). Then Primase can bind and synthesise a primer. The reaction order was probed using antibodies raised to purified dnaB, factor Y and Z proteins (McMacken et al 1977; Ueda et al, 1978).

Unlike G4 and fd, no unique primer is synthesised on ØX174. A yield of 6-8 oligonucleotides per template can be obtained, varying in length from 16-50 residues, and of heterogeneous sequence (McMacken and Kornberg 1978). From the amount of single stranded DNA dependent ATPase activity associated with the intermediate, it was concluded that a single dnaB molecule was present per ØX174 circle. It was suggested that the single dnaB molecule was responsible for all the primers produced on one template, and that its action was that of a mobile promoter for the primase (Kornberg, 1978). In fact, Kornberg (1978) says that the dnaB protein, by moving processively in the 5'→3' direction on the template strand using its single stranded DNA dependent rNTPase activity, acts as the promoter to initiate primer synthesis for Okazaki fragments. As it stands, this system is specific for ØX174 ⊕ strand DNA. For extrapolation to the E. coli chromosome other proteins must be involved.

Clearly these three single stranded bacteriophages have solved the problem of DNA chain initiation in different ways, as summarised in Table III. The reason for this is probably topological, and involves nucleotide sequences at the origin of replication (1.5.5).

Stage	M13	G4	ØX
Prepriming	binding protein	binding protein	binding protein protein i, protein n, protein n , dnaB protein, dnaC protein
Priming	RNA polymerase, discriminating factors	primase	primase, dnaB protein
Elongation	DNA polymerase III holoenzyme	DNA polymeraseIII holoenzyme	DNA polymeraseIII holoenzyme, protein

Table III. Protein Requirements for the In Vitro Conversion of Phage Single-Stranded DNA to Duplex RFII.

1.5.4.2. Priming on double stranded ØX174RFDNA.

For duplex DNA, the production of a 3' hydroxyl end to prime DNA synthesis can be most easily achieved by introducing a nick. ØX174RF replication is the best studied example of this kind of initiation. The 'phage-encoded gene A product is an endonuclease which nicks the ⊕ strand of an RFI molecule (Henry Knippers, 1974). The protein is site-specific, actually nicking within its coding sequence (Ikeda et al, 1976). Gene A protein is found covalently attached to the 5' end of the resulting RFII molecule, and it moves with the replication fork around the circle, cutting the product and sealing it into a circle when a unit length has been made (Eisenberg and Kornberg, 1979). These authors report 1:1 stoichiometry for the geneA:RFII complex, but Ikeda et al (1979) saw multimers of geneA of up to 10 molecules/RFII. The action of gene A is thus more than that of a simple endonuclease, and remains to be solved in detail.

Langeveld et al (1978), showed that gene A protein nicked both ØX174 and G4 RFI DNA in an A:T rich region of 30 nucleotides, which was identical in both. Gene A protein will only nick supercoiled RFI and not relaxed closed circular duplex DNA (RFIV), (Marians et al, 1977), which

suggests that the enzyme may require partially destabilised (i.e. single stranded) regions for endonucleolytic activity. DNA gyrase is necessary to convert RFIV DNA to RFI to act as template for both RF replication and viral strand replication (Marians et al, 1977). Filutowicz (1980) observed the same requirement for in vivo E.coli chromosome replication, indicating that correct superhelicity is probably a general condition for initiation of DNA synthesis.

RF replication of the filamentous 'phages (M13,fd,f1) also depends on a 'phage specified endonuclease, the gene II protein, which nicks only RFI DNA from these 'phages and at a specific site (Meyer and Geider, 1979).

1.5.4.3. Priming on plasmid ColE1 DNA.

ColE1 requires no plasmid coded proteins for its replication (Kahn and Helinski, 1978). Analysis of the products of in vitro replication using E. coli extracts showed completely replicated molecules, and others carrying a small loop in a specific region (Sakakibara and Tomizawa, 1974). The first product of synthesis is a specific piece of one strand the 6S L fragment (Tomizawa, 1975). The 6S L strand is initiated at a fixed point, from one of three residues, dA,dA or dC in a region of 5dA residues bounded by G-C rich regions (Tomizawa et al, 1977). Approaches have been made to discover the minimum sequences, i.e. origin regions, capable of allowing survival of hybrid plasmids (Backman et al 1978; Kahn et al, 1978).

The origin region isolated by Backman et al (1978) was a 580 base pair fragment with only 13 base pairs downstream from the origin site. Conrad and Campbell (1979), showed that an RNA transcript of about 100 nucleotides was transcribed in vitro from this region, starting about 450 base pairs away from the origin. This RNA transcript was incorporated into a model of initiation at the ColE1 origin, "the nomadic

primer model" by Backman et al (1978). It was proposed that the RNA transcript was processed, and then hybridised to the origin region priming DNA synthesis. Conrad and Campbell (1979), strengthened this idea by showing that there was some hybridization of this transcript, and a similar one from the plasmid RSF1030 which bears no extensive sequence homology, to a restriction fragment carrying the origin site. They also showed that extracts prepared from E. coli mutants defective in ribonuclease III did not support ColE1 replication in vitro, but could be complemented by purified protein, suggesting that this enzyme may be responsible for transcript processing. Significantly, mutants with altered copy number control were found to have alterations in this region. Using the sequence data of Bolivar et al (1977) for pBR345, a ColE1-like origin carrying hybrid plasmid, 10 out of 12 bases in a part of the 100 base pair transcript match with a sequence at the origin. Itoh and Tomizawa (1978) showed that in vitro ColE1 replication could be performed using RNA polymerase and ribonuclease H, further evidence for processing of some kind.

This intriguing model for RNA priming remains unproved. Certain features are in its favour, since it attributes a specific role to the transcript, and also explains the "one-off" requirement for RNA polymerase in initiating the 6S L strand (Tomizawa, 1975).

1.5.4.4. Priming in the bacteriophage T4 system.

Bacteriophage T4 replicates using exclusively 'phage encoded proteins. These proteins have been extensively purified, and used to construct an in vitro system capable of replicating double stranded DNA using pre-existing nicks (Liu et al 1978). Seven proteins were identified in this system, two of which, gene 41 protein and protein X, combine to give an RNA polymerase activity involved in priming replication on both single stranded and double stranded DNA templates. Further work has shown that the products of three genes 39, 52 and 60

interact to form a complex which has ATP-dependent DNA topoisomerase activity, (Liu et al, 1979). Mutants in these three genes, detected as "DNA-delay mutants", were shown to be defective in the initiation of DNA synthesis (McCarthy et al, 1976). This enzyme cannot introduce supercoils into the DNA molecules tested as substrates, unlike E. coli DNA gyrase, but is effective at removing them. In a later paper, Liu et al (1980) report that the enzyme can perform a variety of topological functions, including relaxation of supercoils, unknotting of DNA and decatenation. In doing so, it always reduces the linking number of the DNA (Crick, 1976) by integrals of two, possibly by breaking one double helical strand, and passing another through the gap before resealing. Liu et al, (1979) speculated on a model for the involvement of T4 DNA topoisomerase in DNA initiation by its action as a site specific DNA gyrase, introducing negative supertwists at the origin, and allowing it to be primed.

1.5.5. Origin sequences.

Numerous replication origins have now been sequenced, including the origin of complementary strand synthesis of fd (Gray et al, 1978), and G4 (Sims and Dressler, 1978) the E. coli origin (Meijer et al, 1979), ØX174 and G4 RF form origins (Langeveld et al, 1978) as well as the eukaryotic origins of polyoma and SV40 (Soeda et al, 1978). It is now possible to compare structural features in these origins, and predict their functional significance.

Sims et al (1979), sequenced the negative-strand origin regions of 'phages G4, St-1, ØK and α-3 to look for structural similarities, since these 'phages all require dnaG primase for their replication. In all four, the negative strand initiation site lies in an intercistronic region of about 135 bases. Two banks of conserved sequences of 45 bases and 42 bases, separated by a 13 base divergent sequence, were found. The two conserved stretches can be folded into hairpins of similar size,

and the origin lies just prior to one of these hairpins. They posed the question of whether other origin regions, e.g. λ (Hobom et al, 1978), E. coli, (Meijer et al, 1979) bore the same structural features. Hybridization showed that there is no sequence homology between the single stranded phages and the E. coli chromosome. However, there is gross secondary structure similarity between all of the sequenced origins.

The significance of this work is that it may explain the roles of other proteins involved in initiation. The isometric phages studied use only primase whereas ØX174 negative strand synthesis requires the much more complex system described earlier (1.5.4.1.). In turn, synthesis of negative strands of ØX174 does not commence from a specific origin (McMacken and Kornberg, 1978). Clearly the extra proteins remove the necessity to recognise a specific sequence. Sims et al (1979) suggest that this is because the nucleoprotein complexes formed by these additional proteins and DNA mimic the secondary structure recognised by primase. For initiation of Okazaki fragments this is obviously important, since it would be an intolerable strain on an organism to possess real origin sequences at such frequent intervals.

It has also been argued that the structural similarities seen between the primase specific origins and λ and E. coli origins, (Messer et al, 1978) reflect the requirement of primase for synthesis of ori-RNA in all of these systems. RNA polymerase is said to "transcription-ally activate" the λ origin region, rendering it accessible to replication proteins (Hobom et al, 1978). The E. coli origin remains obscure, particularly due to the inability to isolate putative ori-RNA Fujimara et al, 1978). It may prove to be the case that RNA polymerase is not used to synthesis primer RNA for use by the elongation machinery, in which case its role remains to be clarified.

1.5.6. Eukaryotic DNA Replication.

To solve the problem of replicating their vast amount of DNA, eukaryotes initiate synthesis at multiple sites on their chromosomes, from which replication proceeds bidirectionally, (Huberman and Riggs, 1968). The genetic and enzymological details of eukaryotic DNA replication are sparse compared to those of prokaryotes, and consequently reviews have been comprehensive (Edenberg and Huberman, 1975; Sheinin et al, 1979). Comment here will be restricted to a brief discussion of replication proteins, the nature and frequency of replicons, the evidence for RNA priming and the studies on a single replicon (SV40) which may aid understanding of the initiation of DNA synthesis.

1.5.6.1. Eukaryotic replicons.

The size of eukaryotic replicons varies from about 4 μm (1.3×10^4 bp) to 280 μm (90×10^4 bp) (Sheinin et al, 1979). Callan (1974) showed that there were large variations in interorigin distance in DNA from amphibian cells at different stages of development. A large number of apparently synchronous closely spaced initiations can be seen in embryonic S phase cells, whereas those in somatic cells are few and far apart. The autoradiographic work of Blumenthal et al, (1974) on origin frequency in Drosophila melanogaster is of central relevance. They compared rapidly growing embryonic cells with somatic cells from a later developmental stage. In rapidly dividing cells they observed frequent initiations spaced at 3.5 m intervals, which were not present in slowly dividing cells. Such cells had much greater interorigin distances characteristic of the heterochromatisation of their DNA. They suggest that the longer S phase of slow growing cells is due to heterochromatisation of regions of the chromosome, particularly centromeric satellite DNA, which prevents origin regions from being opened, coupled with the uniform rate of fork progression. Eukaryotic

DNA is rich in inverted repeat sequences; they comprise nearly 6% of the human genome (Dott, 1976). At the moment it is mere speculation that these sequences are involved with replicon origins, but such an idea is substantiated by the fact that palindromes are found at the origins of replication of the eukaryotic viruses, SV40 (Subramanian et al, 1977), parvoviruses (small single strand DNA viruses with terminal palindromes which fold back to form hairpins which prime replication, Tattersall and Ward, (1976)), and adenovirus (Kornberg, 1980). The SV40 origin region contains a 27 base pair G/C rich palindrome flanked by A/T rich regions (Subramanian et al, 1977). Shortle and Nathans (1979), using a local mutagenesis technique, have been able to observe profound effects of single base mutations in this region on plaque forming ability and DNA replication. The isolation by Stinchcombe et al, (1979) of an 850 base pair yeast DNA sequence (ars 1) in a 1.4 kb fragment carrying the trip gene, which allows replication of all colinear DNA is further support for the existence of specific origins in eukaryotes.

1.5.6.2. Eukaryotic DNA Replication Proteins.

The list of eularyotic proteins implicated in DNA replication is much less complete than its bacterial counterpart. Three types of DNA polymerase have been discovered in most organisms, called α, β, and γ (Kornberg, 1980). DNA polymerase α and β are nuclear enzymes, and polymerase γ is present in nuclei and mitochondria. Polymerase α appears to be responsible for chromosome replication due to its association with cell proliferation. The levels of polymerase β are enhanced 7-10 fold in neuronal rat nuclei exposed to UV light, suggesting a role in repair (Hubscher et al, 1978). Polymerase γ is responsible for mitochondrial DNA replication, and also adenovirus replication in the nucleus (Arens et al, 1977). Yamaguchi et al, (1980), showed that chick embryo DNA polymerase γ is capable of

synthesising long continuous DNA strands, whereas polymerase α from human KB cells makes short pieces of 11 nucleotides per binding event (Korn et al, 1978). Yeast, typical of other unicellular eukaryotes, possesses only two DNA polymerases, I and II, which most closely correspond to DNA polymerase α of eukaryotes, and DNA polymerase I of bacteria, respectively (Chang, 1977).

Enzymes such as DNA ligase, single strand DNA binding protein, DNA-dependent ATPase, and nicking-closing enzyme (swivelase) have all been sought for and found in eukaryotes. Two distinct DNA ligases have been found (Soderhall and Lindahl, 1976), and the identification, in S.cerevisiae of the cdc9 gene product as a defective DNA ligase (Johnston and Nasmyth, 1978) confirms its involvement in DNA replication.

Proteins analogous to the prokaryotic helix destablizing proteins have been purified from a variety of sources (review Champoux, 1978). Herrick and Alberts (1976) isolated two proteins from calf thymus, UP 1 and "acidic" protein, both of which stimulate their homologous DNA polymerase, as does the protein from the fungus Ustilago maydis, which uniquely shows cooperative binding to single-stranded DNA (Banks and Spanos, 1977).

A DNA-dependent ATPase has been purified from calf thymus by Assairi and Johnston (1979). This enzyme may perform the same function as the bacterial rep protein, coupling ATP hydrolysis to DNA strand separation. Nicking-closing enzymes, alternatively called topoisomerases and swivelases, have been extensively purified from numerous eukaryotes, including S. cerevisiae (Durnford and Champoux, 1978). These enzymes can catalyse the ATP-independent removal of negative or positive supercoils from DNA, using the energy of supercoiling to drive the reaction. They differ from their nearest prokaryotic counterpart, the ω-protein, in several characteristics (Champoux, 1978), most significantly in their ability to remove positive superhelical turns which accumulate ahead of a moving replication form. There have been no

reports of gyrase like activities in eukaryotic nuclei, but such an enzyme may be present in mammalian mitochondria (Castora and Simpson, 1979). It seems unlikely that gyrase exists in nuclei, because the degree of superhelicity of SV40, and by extension, chromosomal DNA, can be entirely attributed to its interaction with histones (Champoux, 1978). Nicking-closing enzyme has been shown to assist the in vitro formation of nucleosomes from purified DNA and histones under physiological conditions (Germond et al, 1979). Thus, this enzyme may have a role in chromatin assembly in vivo.

1.5.6.3. RNA Priming in Eukaryotes.

The involvement of RNA at replication origins has yet to be proved, but the work of Guy and Taylor (1978), gives a positive indication of this. Using cells blocked at the G1/S boundary by fluorodeoxyuridine, they found that new origins accumulated but these could not be used (as measured by the release of nascent fragments), if actinomycinD was added before a ^{3}H-thymidine pulse label. They also concluded that Okazaki piece formation was not inhibited, since replication forks in progress could be extended in the presence of actinomycinD. Thus it would appear that actinomycinD specifically inhibits initiation without affecting ongoing replication, implying an involvement of RNA synthesis at replication origins. However, such data must be interpreted with caution, since it is based on assumptions of the modes of action of the two inhibitors. There is good evidence that RNA priming of nascent fragments occurs, from nearest neighbour analyses which establish RNA/DNA junctions. Kowalski and Denhardt (1979) have shown this in 3T6 cells. Eliasson and Reichard (1979) studied initiator RNA synthesis in isolated nuclei from polyoma infected cells and produced evidence for a decanucleotide primer, the synthesis of which was totally resistant to α-amanitin. Together with the utilisation of dNTP's almost as well as rNTP's, this

suggests the existence of a mammalian counterpart of the dnaG gene product of E.coli. This concurs with the results of Guy and Taylor (1978).

Certain eukaryotic viruses use highly specific methods for priming their own replication. RNA tumour viruses use a tRNA present in virion particles to prime synthesis by reverse transcriptase (Harada et al, 1979).

1.5.6.4. Replication of SV40.

A few hours after infection of permissive hosts by SV40, a characteristic protein, the T-antigen, is produced. This protein is encoded by the A gene of the virus (review Weinberg, 1977). The role of T-antigen has attracted much attention in recent years. Temperature-sensitive mutations in the A gene were shown to result in a defect in the initiation of DNA replication (Tegtmeyer, 1972; Chou et al, 1974). T-antigen is an autogenously regulated protein of 96,000MW (Tegtmeyer et al, 1975). Its appearance corresponds to the induction of cellular DNA synthesis in the host (Graessmann and Graessmann, 1976). By fluorescence methods, D'Alisa and Gershey (1978) showed Tantigen binding to host DNA in vivo. The origin containing region of SV40 binds T-antigen tightly (Tjian, 1978). Such an interaction was confirmed by Shortle et al (1979), who used previously generated ori mutants of SV40 (Shortle and Nathans, 1979), and looked for pseudorevertants that retained the defective origin. Second site mutations were shown to lie in the region coding for T-antigen. A stretch of DNA 75-400 bases long in the origin region appears to be specifically exposed to endonuclease action (Varshavsky et al, 1978).

The mode of action of T-antigen is still undiscovered. Tjian and Robbins (1979) isolated an SV40 T-antigen related protein from cells infected with an adenovirus-SV40 hybrid virus. The protein, 107,000MW, mostly coded by SV40, was shown to contain ATPase and protein kinase

activities, which were separable by ion-exchange chromatography. At the moment, it is not known whether the SV40 T-antigen itself is responsible for either of these activities. T-antigen is probably more than a site-specific endonuclease, since this activity could be detected fairly easily. Unfortunately, initiation seems to be one of the few features which current in vitro systems are incapable of performing (Su and DePamphilis, 1978).

1.5.7. Summary.

This section has been concerned with the molecular events which bring about DNA chain initiation. Various prokaryotic systems achieve this feat by one, or a combination of the following processes: a. recognition of specific origin sequences; b. unwinding to expose single stranded regions; c. endonucleolytic cleavage to produce 3'OH ends; d. priming of replication by RNA. These generalities may prove useful in looking at the problem in eukaryotes.

This discussion has avoided reference to the possible site of synthesis of DNA. There is evidence that the bacterial chromosome is associated with the cell membrane, from electron microscopy (Siegel and Schaecter, 1973) and through the isolation of folded chromosome/cell envelope complexes (Drlica et al, 1973). Linkage of the B.subtilis chromosome to membrane components may occur at the replication origin (Seiki et al, 1979). Gudas et al (1976) reported an outer membrane protein (protein D) in E.coli, whose synthesis was intimately linked to the initiation of DNA synthesis. But the postulated interaction between DNA and membrane is difficult to prove, and must be regarded as unresolved. Certainly, as far as eukaryotic DNA replication is concerned, when Edenberg and Huberman (1975) reviewed the subject, there was no evidence to suggest a membrane involvement. Replication takes place throughout the nucleus in proportion to DNA concentration.

1.6 Saccharomyces cerevisiae

The budding yeast Saccharomyces cerevisiae is a unicellular eukaryote. It possesses subcellular organelles such as a nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus, which clearly distinguish it from prokaryotes (Matile et al, 1969). Yet it has a low DNA complexity, 9×10^9 daltons DNA/haploid nucleus (Carter, 1975), barely four times that of E.coli. For genetic studies the main advantage of S.cerevisiae lies in its ability to exist in either the haploid or diploid state (Fig.6). In homothallic strains, the haploid exists as one of two mating types, either a or α (Lindegren and Lindegren, 1943). Opposite mating types can fuse to form a diploid, which is quite stable and grows vegetatively in the same way as haploids. Under appropriate conditions, such as nutrient starvation, a diploid can be induced to sporulate and enter meiosis, giving rise to an ascus containing four spores. It is possible to dissect asci and analyse separately the four products of meiosis (Mortimer and Hawthorne, 1969). Thus mutants can be easily obtained in the haploid state, and studied by complementation in diploids.

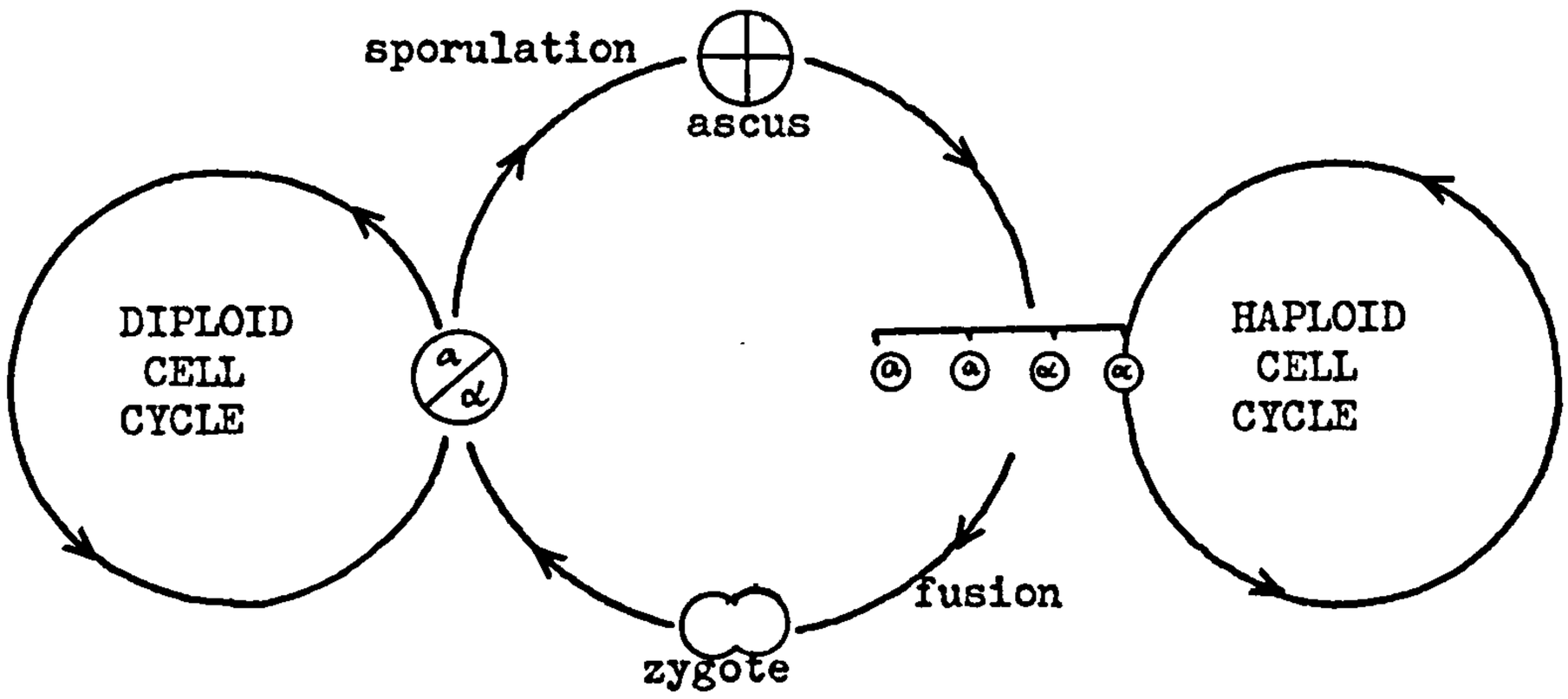

Fig.6: Life Cycle of S.cerevisiae.

The morphological events of the cell cycle have been well characterised (Hartwell, 1974). Byers and Goetsch (1973), in an elegant electron microscopic study, followed the duplication of spindle plaques in relation to the cell cycle. These structures are dense microtubule organising centres located in the nuclear membrane. Spindle plaque duplication occurs at the same time as bud emergence, and is the first definite characteristic of a new cell cycle. In later stages of the cycle, the plaques separate (remaining in the nuclear membrane) and a mitotic spindle forms between them. (Fig. 7).

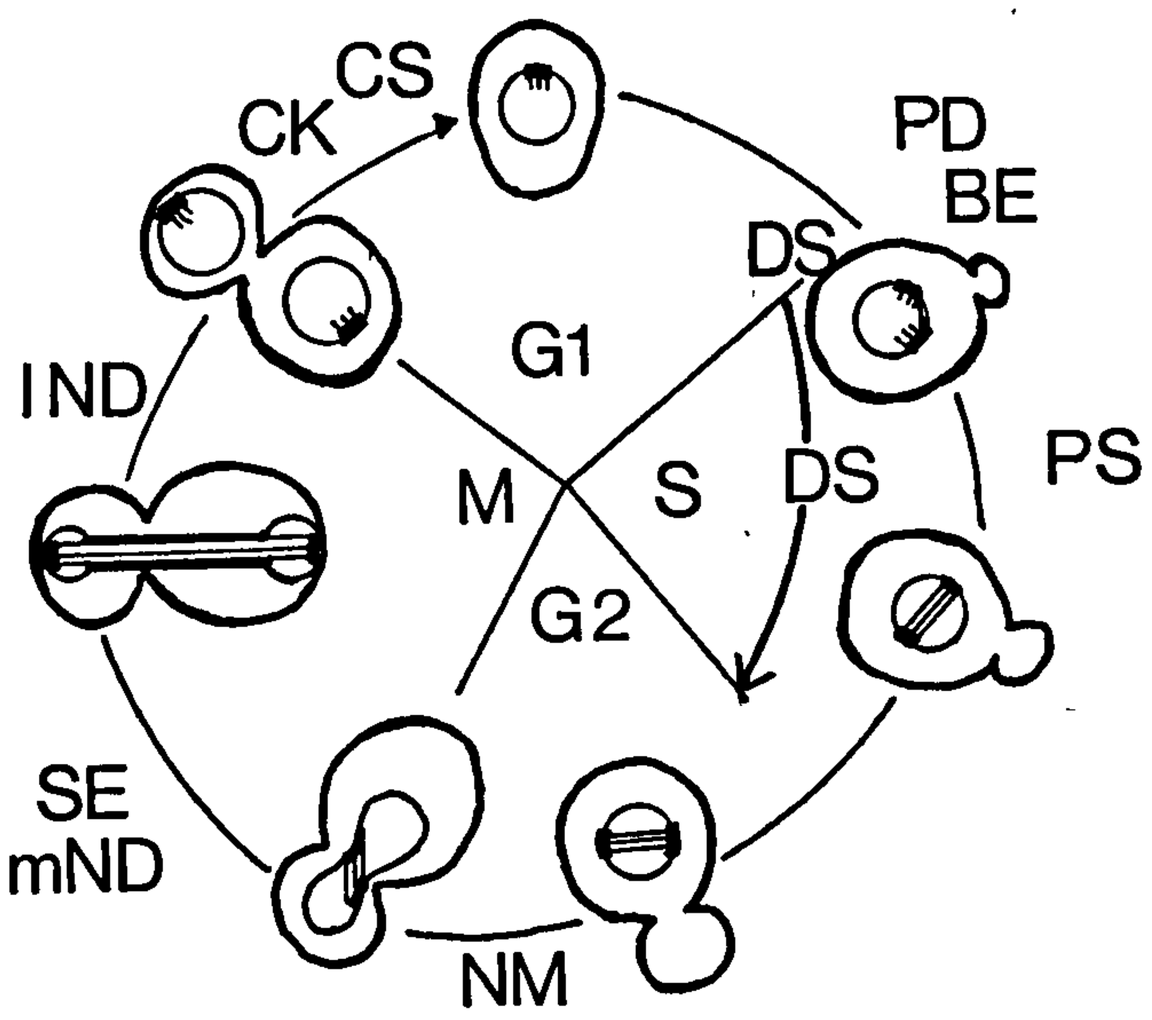

Fig. 7: Landmarks of the S. cerevisiae cell division cycle. Abbreviations: PD, plaque duplication; BE, bud emergence; iDS, initiation of DNA synthesis; DS, DNA synthesis; PS, plaque separation; NM, nuclear migration; mND, medial nuclear division; SE, spindle elongation; lND, late nuclear division; CK, cytokinesis; CS, cell separation. Distance between events does not necessarily reflect interval of time between events.

Hartwell (1967) produced a series of 1500 temperature sensitive mutants from a strain of S. cerevisiae, A364A. Of these, 148 were termed cell division cycle (cdc) mutants, since they arrest with a characteristic morphology, called the "terminal phenotype" after a shift to the non-permissive temperature. The permissive temperature for these strains is 23°C and the restrictive temperature 36°C or 38°C, depending on the leakiness of the particular lesion. Genetic analysis

shows that these 148 mutants fall into 32 complementation groups, presumably defining 32 genes whose products are required for traversal of the cell cycle (Hartwell et al, 1973). Much work has gone into the characterisation of these mutants in the last few years. Hartwell (1971, 1973) showed that cdc 8 and 21 were defective in the elongation reactions of DNA synthesis, and cdc 4, 7 and 28 prevented the initiation of replication. Byers and Goetsch (1973) characterised the mitotic spindle and plaque morphology of the mutants at the restrictive temperature. In work previously described (1.2.2), Hereford and Hartwell (1974) sequenced the steps mediated by α-factor, cdc 28, cdc 4, and cdc 7 in the prereplicative phase of the cell cycle. Subsequently, Hartwell (1976) characterised cdc2 and cdc6 with respect to the hydroxyurea sensitive steps (i.e. DNA synthesis) using reciprocal shift experiments.

Hartwell et al, (1974), compiled this data into a map of gene function in the yeast cell cycle, which distinguished two dependent pathways. One pathway is composed of plaque duplication, plaque separation, initiation of DNA synthesis, DNA synthesis, medial nuclear division, late nuclear division and spindle elongation, cytokinesis and cell separation. The second involves bud emergence, nuclear migration, cytokinesis and cell separation. Only completion of the first pathway is required to enter a new cell cycle, since one mutant cdc 4, shows multiple cycles of bud emergence. These features are shown in Fig. 8 (Simchen et al, 1978).

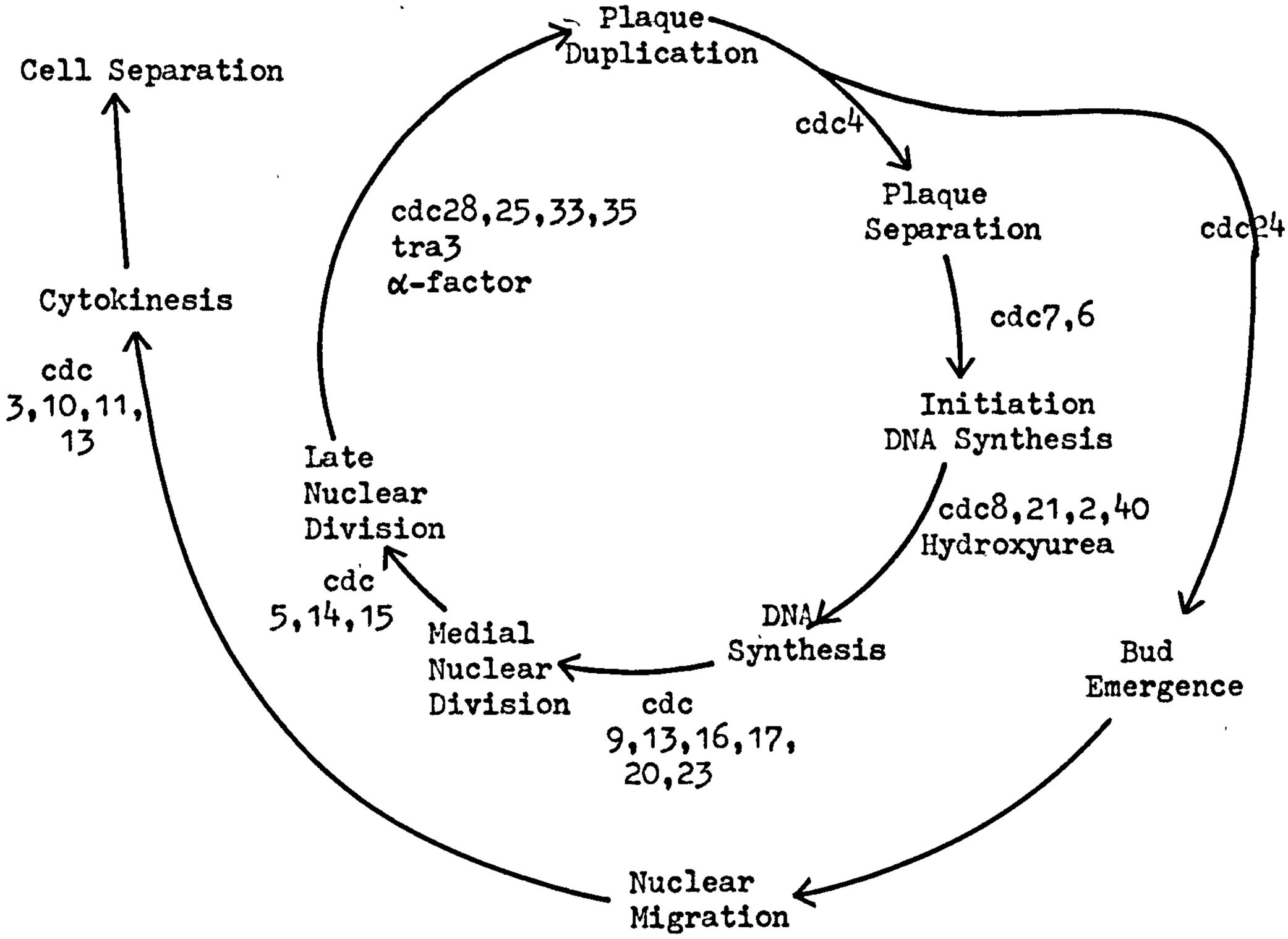

Fig. 8: Gene functions in the Cell Cycle of S.cerevisiae (from: Simchen, 1978).

The mutant collection has now been extended, Kassir and Simchen (1978) isolated cdc40, defective in DNA replication. A few mutants have been characterised, mainly those involved with ongoing DNA replication. Game (1976) and Bisson and Thorner (1977) confirmed that the DNA elongation mutant cdc21 was defective in thymidylate synthetase. The cdc9 lesion, (and its counterpart cdc17 in Schizosaccharomyces pombe, (Nasmyth, 1979)), has been shown to result in a defective DNA ligase (Johnston and Nasmyth, 1978). Prakash et al, (1979) have observed decreased UV mutagenesis in cdc8, and suggest that the CDC8 gene product plays a direct role in error-prone repair. The tra3 mutant, causing derepression of the biosynthetic enzymes for histidine, lysine, arginine and tryptophan (Wolfner et al, 1975), was shown to arrest at "Start" (1.2.2.). Though not isolated as a cell

cycle mutant, it possesses the desired characteristics.

There are probably many more genes controlling cell cycle events which have not, or cannot, be detected in temperature-sensitive mutations (Hartwell, 1974). At the moment, the goal is to understand the available mutants.

S. cerevisiae has been shown to contain a 2μm circular plasmid, present in about 60-100 copies per cell (Guerineau et al, 1971; Livingston, 1977). Its replication is under the control of the cdc genes (Chapter 6; Livingston and Klein, 1977). Of unresolved function, this plasmid is proving useful as a eukaryotic vector in gene cloning (Beggs, 1978), and as a model replicon for use in in vitro DNA synthesising systems (Chapter 6; Jazwinski and Edelman, 1979).

1.7. Aim of this thesis.

Hereford and Hartwell (1974) showed that the S. cerevisiae strain cdc7.4 (H201.14.4), defective in the initiation of DNA synthesis, did not require protein synthesis to perform DNA replication when blocked cultures were transferred to the permissive temperature. This suggests that the lesion may lie in a component of the replication apparatus itself, or in some factor which functions immediately before replication. This work has been undertaken to attempt to discover the molecular basis of the mutation in this strain, since such knowledge will illuminate the problems of DNA replication and its control and coordination during cell division. The work has involved (i) a further biochemical characterisation of cdc7.4, and other cdc mutants, obtained from L. H. Hartwell; (ii) comparison of the protein labelling patterns of cdc7.4 with other strains including other alleles of cdc7, and further genetic analysis of the temperature-sensitive lesion (iii) assays of suspected temperature sensitive activities in cdc7, (iv) a study of the replication of the 2μm yeast plasmid in initiation

defective mutants, and (v) attempts to construct <u>in vitro</u> assay system to assist in isolation of the CDC7 gene product.

CHAPTER TWO

General Materials and Methods

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2.3 Media

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2.6 Radioactive incorporation into macromolecules

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CHAPTER TWO.

GENERAL MATERIALS AND METHODS

2.1 Materials.

The following radiochemicals were used : 6-^{3}H uracil (21-30 Ci/mmole); 6-^{3}H thymidine (24 Ci/mmole); ^{3}H amino acid mixture (code number TRK 440); (U-^{14}C) protein hydrolysate, 59mCi/matom carbon); (2-^{14}C) uracil/60mCi/mmole; (methyl-^{3}H) thymidine 5^{1}-monophosphate (54Ci/mmole); Deoxy(8-^{3}H) guanosine 5^{1}-triphosphate (10.6Ci/mmole); methyl-^{3}H) thymidine 5^{1}-triphosphate (30-47Ci/mmole); (5-^{3}H) uridine 5^{1}-triphosphate (20Ci/mmole); uridine 5^{1}-(α-^{32}P) triphosphate (400Ci/mmole); ^{32}P orthophosphate in dil. HCl pH2-3 (115-135 Ci/mg P); 98% sulphuric acid ^{35}S (22mCi/mmole). All were obtained from the Radiochemical Centre, Amersham.

Nucleotides were obtained from Boehringer Mannheim, Germany.

Calf Thymus DNA (type I), Horse heart cytochrome C (type III), Dexyribonuclease I, Ribonuclease A, PMSF, Cycloheximide, were obtained from Sigma.

Yeast extract, Bacto-peptone, Bacto-typtone and Noble agar were obtained from Difco.

DEAE-cellulose (DE52), Phosphocellulose (P11) and cellulose CF11 were obtained from Whatman. DEAE-Sephadex A25 was from Pharmacia.

Bovine serum albumin, Ethidium bromide, N-hydroxyurea were obtained from BDH. Caesium chloride was from Fisons.

Thiolutin was a gift from Nathan Belcher, Pfizer Inc.

All other chemicals were AR grade.

2.2 Yeast strains.

The following strains were obtained from L.H. Hartwell, University of Washington, Seattle; X2180-1A (a, prototrophic); X2180-1B (α, prototrophic); S2072D (α, arg4, leu 1, thr 1, trp 1); cdc28.1, H185.3.4 (a, ade 1(2), ura 1, his 7, lys 2, tyr1, leu 1); cdc21.1

H.146.2.3. (a,adel(2), ura 1, lys 2,); A364A (a, ade 1(2), ura 1, his 7, lys 2, tyr 1); the following cdc mutants are isogenic with A364A: cdc4.3 (H135.1.1), cdc7.4 (H201.14.4 and 4008, both have the same characteristics), cdc28.2 (E420), cdc8.3 (13052), cdc7.1 (124), cdc7.2 (325), cdc7.3 (18032), cdc7.7 (E247).

The dTMP permeable strain g308-6C (a, lys , his , ilv, tmp1, tup) was obtained from Dr. J. G. Little. Commercial yeast was supplied in 1kg slabs by UCL Refectory, or in 50 litres primary fermentation (Strain 1318) from Youngs Brewery, Wandsworth.

The following strains were generated during the course of this work cdc7.4ℓ(a, ade 1(2), ura 1, his 7, lys 2, tyr 1); cdc7.4, DE200.1.3 (a, leu 1, arg 4); cdc7.4, DE200.3.2 (α, ura 1, his 7, leu 1); cdc21.1, DE46/2 (a, ade 1/2), ura 1, lys 2, tup).

2.3 Media.

MVmedium : Per litre, this contained :- 20g glucose, 6.7g Yeast Nitrogen Base (without amino acids). Yeast Nitrogen Base was obtained from Difco, and sterile filtered into the autoclaved medium or made in the laboratory to the following composition :

Boric acid 500μg, $CUSO_4 \cdot 5H_2O$, 40μg, KI 100μg, $FeCl_3$ μ200 g, $MnSO_4 \cdot 4H_2O$ 400μg, Na Molybdate 200μg, $ZnSO_4 \cdot 7H_2O$ 400μg, (all of these were stored at room temperature as 1000x concentrated solutions, 1ml of each being added per litre of medium); Ammonium sulphate 5g, KH_2PO_4 1g, $MgSO_4 \cdot 7H_2O$ 0.5g, NaCl 0.1g, $CaCl_2$ 0.1g. The following compounds were made up as a mixed solution, 100x concentrated, which was stored frozen at -32°C: 10ml was sterile filtered into the autoclaved medium when cool :- Biotin 2μg, calcium pantothenate 400μg, Folic acid 2μg, inositol 2000μg, nicotinic acid 400μg, p-aminobenzoic acid 200μg, pyridoxine HCl 400μg, riboflavin 200μg, thiamine HCl 400μg.

40mg/litre amino acids were added as required.

(YE)SD-All medium : Per litre, this contained :- (1g yeast extract).

20g glucose, 10g succinate, 6gNaOH, 6-7g Yeast Nitrogen Base (made as under MV medium), 40mg histidine, lysine, tyrosine (leucine), 10mg adenine, 10mg or 5mg uracil (the subscript denotes the amount added). Final pH 5.8.

YEPD-AU medium : Per litre, this contained:- 10g yeast extract, 20g Bacto-peptone, 20g glucose, 40mg adenine, 40mg uracil. If necessary, 40mg threonine was sterile filtered and added when cool. With 20g per litre agar (labM) added, this medium was used for YEPD-AU agar plates and slopes.

CAD medium : Per litre, this contained:- 2g Casamino acids (Difco), 20g glucose, 6-7g Yeast Nitrogen Base, 50mg adenine.

"L Broth" medium : Per litre, this contained:- 10g Bacto-tryptone, 5g yeast extract, 5g NaCl, 1g glucose.

All media were autoclaved at 120°C for 15 minutes.

2.4 Storage of strains.

For routine use, strains were maintained on YEPD-AU agar slopes in 5ml Bijou bottles at 23°C, restreaking every 2 months. For long term storage, two methods were used; (a) suspensions in evaporated milk were dried onto 1cm squares of Whatman No.4 filter paper and stored at -32°C (Method of Yeast Genetics Stock Centre, C.R. Contopoulos, pers. comm.); (b) suspensions of stationary phase cells in 15% glycerol were stored at -70°C (L.H. Hartwell, pers. comm.).

2.5 Methods for monitoring cell number.

Two methods were used: (a) Haemocytometer. Measurements were made using an Improved Neubauer haemocytometer (Hawksley). Culture samples were fixed in an equal volume of 7.4% formaldehyde. Cells carrying buds were counted as 2 cells when the bud exceeded half the size of the mother cell (Hartwell, 1974).

(b) Light scattering. Routinely cell densities were measured by reading optical densities at 660nm from a standard

curve which had been calibrated by haemocytometer. This method was satisfactory for exponential cultures only. Cells show a marked reduction in volume upon entering stationary phase and the OD_{660} relationship ceases to hold. Also, cultures which have been blocked by temperature show volume increases, and consequent OD_{660} increases, without cell division.

Cell viabilities were performed by diluting 0.1ml samples of culture into 0.3ml sterile water, and sonicating on an MSE sonicator for 4 seconds at full pitch (this does not affect cell viability, W.F. Wakeling pers. comm.). Two 50 fold serial dilutions were performed in sterile water, and 0.1ml of diluted cells were plated on YEPD-AU agar plates. 50 colonies growing after 2-3 days at 23°C correspond to $5x10^6$ viable cells/ml in the original culture.

2.6 Radioactive incorporation into macromolecules.

2.6.1 DNA synthesis.

Unless otherwise stated, 0.5ml samples of culture radioactively labelled using 6-^{3}H uracil were dispensed into 1ml 2N NaOH, mixed and incubated at 60°C for 3 hours. This incubation was found to be necessary in order to reduce contaminating RNA counts to a minimum. Samples were chilled in ice and 0.5ml 0.2mg/ml carrier calf thymus DNA was added, and the DNA precipitated by the addition of 1ml ice-cold 50% TCA. Prolonged mixing was required here. Samples were filtered through Whatman GF-C 2.5cm circles, washed with 5% ice-cold TCA and absolute ethanol. The filters were dried under an infra-red lamp and counted in Brays scintillation fluid (2.6.4).

2.6.2 RNA synthesis.

0.1ml samples of culture were arrested with 0.1ml 7.4% formaldehyde and stored at room temperature until processed. They were then chilled and precipitated by the addition of 1.5ml 5% TCA (ice-cold), and filtered as in 2.6.1.

2.6.3 Protein synthesis.

0.5ml samples of culture were added to 0.5ml 10% TCA in glass tubes, and heated at 90-95°C for 30 minutes before filtering as in 2.6.1.

2.6.4 Scintillation counting.

Radioactivity was measured using Beckman LS200 or LS355 scintillation counters, possessing comparable counting efficiencies. The scintillation fluid was modified Brays solution (15g BBOT, 1.5 litres toluene, 200g naphthalene, 1 litre 2-methoxyethanol) or PPO/POPOP (10g PPO, 1g POPOP, 2.5 litres toluene).

Mixed ^{14}C and ^{3}H counts were separated by external standard method using quenched ^{14}C Packard samples (100,000dpm/pot) for calibration.

2.7 Chemical estimation of macromolecules.

DNA was estimated by the Burton (1956) diphenylamine reaction. The procedure used a 1:1 sample to reagent ratio, and was performed on 1ml 10% perchloric acid hydrolysates as described by Kay (1973).

RNA was estimated in the perchloric acid hydrolysates using the orcinol reaction described by Hutchinson and Munro (1961).

Protein was determined by the method of Lowry et al (1951), using bovine serum albumin as standard, or by a U-V method (Layne, 1957).

2.8 Preparation and assay of α-factor.

2.8.1 Preparation of α-factor. (Duntze et al, 1973).

A 20 litre aspirator containing 18 litres of MV medium was equilibrated overnight at 30°C. A flow of air was sterilized by passing sequentially through a non-absorbent cotton wool filter, a 12.5% sulphuric acid wash, a distilled water wash, and a sterile filter (Whatman Gamma 12 in line filter unit, grade 12-10 filter, 1μm retention) and bubbled from the bottom of the aspirator to exit through a similar

filter unit (grade 12-80 filter, retention 8μm) at a flow rate of 2 litres/minute. The exit filter was removed temporarily to allow a 90ml innoculum of S.cerevisiae strain X2180-1B (α mating type) at $2x10^7$ cells/ml to be added. Experiments showed that the optimum growth time for maximum α-factor production was 40 hours at 30°C using this innoculum. Beyond this, α-factor activity was rapidly lost. Tanaka et al, 1977 confirmed this observation.

The culture (pH2.8) was filtered through a Whatman GF-C 15cm circle on a Buchner funnel, and the filtrate was loaded onto a 6x20cm Amberlite CG-50 column at 50ml/minute at room temperature. The column was washed with 1 litre of 50% ethanol and α-factor was eluted with 1.5 litres of 80% ethanol containing 0.01MHCl. The eluate was rotary evaporated (water bath at 38°C) to 300ml, diluted 3 fold with water and rotary evaporated again. The pH was adjusted to 5.5 with ammonia solution, stood in ice for 1 hour, and centrifuged in an MSE18 centrifuge at 15Krpm for 15 minutes at 4°C. The supernatant was lyophilized and the resulting solid extracted with 25ml methanol by stirring for 2 hours at room temperature. The solid residue recovered on centrifugation is an MSE centrifuge at 12Krpm for 15 minutes at 20°C was extracted twice with 15ml methanol. The extracts were pooled and rotary evaporated until small amounts of solid began to appear, then diluted with 300ml distilled water and lyophilized again.

The methanol extraction of the lyophilized solid was repeated as before, but using 12ml and 6ml successively. The supernatants were pooled and assayed for α-factor activity.

2.8.2 Assay of α-factor activity:

(a) Plate assay.

A loopful of a culture of X2180-1A (a mating type) grown in YEPD medium to $2x10^6$ cells/ml was streaked on a YEPD agar plate adjacent to a well bored into the agar using a 0.8cm sterile cork borer. 200μl of

diluted α-factor was added to the well, and the plate incubated at 23°C for 4 hours. α-factor activity was measured microscopically by the incidence of "schmoos" (3.3.1). The minimum α-factor activity required to produce an effect was defined as 1 unit of activity. This assay suffers from subjective errors.

(b) Radioactive assay.

A more direct assay measured the concentration of α-factor required to inhibit nuclear DNA replication. 6x4ml cultures of A364A in YESD-AU_5 at $5x10^6$ cells/ml received additions of 0, 10, 20, 40, 60 and 80 µl α-factor in methanol, and 80, 70, 60, 40, 20 and 0 µl methanol respectively. After 3 hours at 23°C, 6-^{3}H uracil was added to each flask to a final 4 µCi/ml, and growth continued for a further 2 hours. 4ml 2N NaOH was added to each culture, and then left overnight at 37°C. The samples were chilled in ice, and 4ml ice-cold 50% TCA was added to each to precipitate DNA, which was filtered and processed as in 2.6.1. A typical result is shown below.

% α-factor	0	0.25	0.5	1.0	1.5	2.0
^{3}H cpm incorporated	6700	3020	1730	430	200	220

1.5% α-factor was used as the effective concentration in this case. The presence of methanol in the growth medium exerted no effect on the cells as long as adequate glucose (2%) was present. The protein profile on an SDS-polyacrylamide gel of cells grown in the presence of 0-4.7% methanol showed no detectable change, even at the highest methanol concentration.

2.9 Methods for the formation of yeast spheroplasts.

2.9.1 Snail-gut juice (β-glucuronidase) method.

Log-phase cells at approximately $2x10^7$ cells/ml in YESD-AU_{10} were harvested and washed in 1M sorbitol. 2% β-glucuronidase (Glusulase, snail-gut juice; Sigma) in 1M sorbitol was passed through a 0.45 µm Millipore filter (HAWP02500). Cells were resuspended in this solution

to a final density of 1-2x10^8 cells/ml, and incubated at room temperature with occasional agitation until spheroplasting was complete. Spheroplast formation was monitored either microscopically by susceptibility to osmotic lysis on a microscope slide, or by the reduction in OD_{660} after treating with 1% nonidet detergent. Spheroplasts were washed by centrifugation through 1·2M sorbitol in an MSE bench centrifuge at speed 5 for 5 minutes.

2.9.2 Preparation of enzyme from Arthrobacter luteus.

Arthrobacter luteus (Kitamura et al, 1971) was grown in the following medium : per litre, 1g KH_2PO_4, 1g$MgSO_4$-$7H_2O$, 20g yeast cells, 1g glucose, 0.5g yeast extract (Difco), 0.5g ammonium sulphate, final pH8-5. Bacteria were maintained on 2% agar slopes containing this medium. Starting cultures were subcultivated 3 times at 30°C to ensure healthy growth (the final colour of the medium was bright yellow). 25ml starting culture was innoculated into 1 litre of medium, and grown at 30°C for 36 hours. Cells were removed by centrifugation at 10K rpm for 10 minutes at 4°C in an MSE18 centrifuge. The supernatant (containing the enzyme activity) was lyophilized. The product was dissolved in distilled water and dialysed against water to remove salt, then lyophilized again. Finally, the enzyme was dissolved in a small volume (25ml) of 0.1M KH_2PO_4 pH7-5, centrifuged at 10K rpm for 10 minutes at 4°C in MSE18, then dispensed into 1ml aliquots and stored frozen at -32°C.

2.9.3 Use of Arthrobacter enzyme for spheroplast formation.

Yeast cells, preferably in log-phase, were washed in 1.5M sorbitol 5mMEDTA. 3 volumes of cells which had been resuspended in this solution at 10^9 cells/ml, were mixed with 1 volume of enzyme solution, and 10 µl β-mercaptoethanol, and incubated at room temperature for 15 minutes - 1 hour. Spheroplast formation was determined as in 2.9.1.

2.10 One dimensional SDS-polyacrylamide gel electrophoresis of proteins.

The method and apparatus used was that of Studier (1973). Solutions for SDS gels were stored at 4°C, and stock buffer solutions (2) and (3) were autoclaved. The stock solutions were : (1) Acrylamide/Bisacrylamide (30:0.8). The bisacrylamide was weighed accurately to within 1mg. (2) 1.5M Tris-HCl pH 8.8, (3) 0.5M Tris-HCl pH 6.8.

Routinely, a 10% separating gel with a 5% stacking gel was used. Spacers between the glass plates were 0.8mm x 1cm teflon strips. The separating gel was 13.5cm long. Well formers with either thirteen 0.8cm teeth or twenty 0.4cm teeth were used, and the stacking gel was 1cm from the bottom of the sample wells to the top of the separating gel. The glass plates were clamped with bulldog clips and the edges sealed with 2% agar.

The following quantities were used to make one separating gel : 10ml acrylamide/BIS stock solution, 3ml 1.5MTris-HCl pH 8.8, 0.3ml 10% SDS, 0.6ml 0.1M EDTA, 15.8ml double distilled water, 14 µl TEMED, 0.3ml 10% ammonium persulphate (prepared freshly). The solution was filtered through a Whatman GF-C 2.5cm circle and degassed before addition of the last two items.

One stacking gel was composed of : 1.66 ml acrylamide/BIS stock, 1.25ml 0.5M Tris-HCl pH 6.8, 0.1ml 10% SDS, 0.2ml 0.1M EDTA, 6.65ml double distilled water, 5 µl TEMED, and 0.1ml 10% ammonium persulphate.

The sample buffer contained 10ml 0.5M Tris-HCl pH 6.8, 10ml 10% SDS, 2ml 0.1M EDTA, 1ml β-mercaptoethanol, 20ml glycerol and 58ml double distilled water. Samples were heated in this buffer at 100°C for 2 minutes.

The electrode buffer (final pH 8.3) contained (per litre): 6g Tris base, 28.8g glycine, 10ml 10% SDS, 2ml 0.1M EDTA.

Samples (20-50 µl, containing 1 µl bromophenol blue) were run in at low current (∿ 12 mamps). Overnight electrophoresis (16 hours) was carried out at 40 volts using 10% gels.

After electrophoresis, gels were stained in 25% isopropanol, 10% acetic acid, 0.05% Coomassie Brilliant Blue, CBB (Fisons) for at least 8 hours at room temperature with constant stirring, then destained overnight in 10% isopropanol, 10% acetic acid, 0.0025% CBB. Destaining was continued in 10% isopropanol, 10% acetic acid until the background was clear.

Gels were dried down onto Whatman 3MM paper, using a gel dryer developed in the laboratory. The gel dryer was attached to a vacuum pump and inverted over a boiling water bath for 45 minutes.

2.11 Preparation of column chromatographic materials.

All columns were run in the cold room at 4°C, using an LKB fraction collector, the eluate being monitored at 280nm in a flow cell.

DEAE cellulose, DE52, powder was defined by decantation, and washed in 1M NaCl. The alkali-acid wash cycle consisted of washing in 0.1M NaOH for 30 minutes, filtering on a Buchner, and washing with water to neutrality. It was then washed with 0.1M HCl for 30 minutes, water, 10x concentrated running buffer, and finally washed and resuspended in running buffer. The suspension was degassed and columns were poured at room temperature.

DEAE Sephadex A25 and Phosphocellulose P11 were treated similarly.

DNA cellulose, both single-stranded and native DNA cellulose were prepared using Calf Thymus DNA and Whatman CF11 cellulose by the method of Alberts et al (1968) by I. R. Johnston. When not in use columns were stored at -32°C in 2M NaCl. Recycling involved extensive washing with 2M NaCl followed by running buffer.

Salt concentrations were determined by conductivity measurements using a Radiometer conductivity meter.

2.12 Preparation of whole cell extracts for enzyme purification.

Cultures were grown to mid log-phase (10^8 cells/ml) in YEPD-AU medium (4 litres yields 20-25g wet weight of cells). Harvesting was

performed either by centrifugation in an MSE Mistral for 10 minutes at 3K rpm at 4°C, or by filtration through Whatman GF-C (15cm circles). Cells were washed once in extraction buffer, EB (50mH Tris-HCl pH8.0, 1mM EDTA, 10% v/v glycerol, 2M NaCl, 1mM PMSF and 2mM DTT), their wet weights measured, and then transferred to 2 Braun homogenizer bottles, each containing 50g acid-washed 40 mesh glass beads, using 6-10ml of EB. A minimum of buffer should be used to yield maximum cell breakage. Cells were broken by 6x10 second bursts in a Braun homogenizer (Botany and Microbiology Dept., UCL), cooled by CO_2. The extract was removed by washing with EB, decanting from the glass beads. One-half volume of 30% Polyethylene glycol (PEG) 6000 in EB was added, and stirred vigorously for 30 minutes in ice. The PEG precipitate, containing all the nucleic acid and 75% of the protein, was removed by centrifugation at 10K rpm for 10 minutes at 4°C in an MSE18, and the supernatant was dialysed for 5 hours against 1 litre 20mM Tris-HCl pH 8.0, 0.5mM EDTA, 10% v/v glycerol, 1mMDTT, 0.1mM PMSF (TEDGP buffer), changing the buffer every hour. The extract was clarified by centrifugation at 10K rpm for 10 minutes at 4°C in an MSE18, and used immediately.

2.13 RNA polymerase assays.

2.13.1 Shultz and Hall (1976).

The reaction mix contained 50mM Tris-HCl pH7.9, 1.6mM $MnCl_2$, 0.5mM ATP, CTP and GTP, 0.02mM UTP, 100 µg/ml (1.1) native: denatured Calf Thymus DNA, and 10 µCi/ml (5-^{3}H)UTP. 50 µl mix was reacted with 50 µl enzyme fraction for 20 minutes at specified temperatures. Reactions were terminated with 0.5ml ice-cold 0.1M sodium pyrophosphate, and 3ml ice-cold 5% TCA. After 15 minutes in ice, samples were filtered through Whatman GF-C 2.5cm circles, washed with ice-cold 5% TCA and then ethanol, dried under an infra-red lamp and counted in Brays scintillation fluid (2.6.4).

2.13.2 Valenzuela et al, (1978).

The reaction mix contained 0.15M Tris-HCl pH 8.0, 5mM $MnCl_2$, 1.5mM ATP, GTP and CTP, 31 μM UTP, 1mM β-mercaptoethanol, 1mg/ml Calf Thymus DNA (native) and 25 μCi/ml (5-^{3}H) UTP. Reactions were carried out using 20 μl mix, 20 μl enzyme fraction, and 20 μl of an appropriate KCl solution. The salt optima for yeast RNA polymerases I, II and III are respectively 0.1M, 0.25M and 0.1 - 0.4M KCl at saturating amounts of native DNA (Valenzuela et al, 1978). Reactions were terminated and processed as in assay (I).

2.14 DNA polymerase assay.

The assay has been described previously (Holmes, 1974). Assays (100 μl) contained 60mM Tris-HCl pH7.8, 0.2mg/ml activated Calf Thymus DNA, 0.6mM dATP, dGTP, dCTP and TTP, 5mM $MgCl_2$, 0.5mg/ml bovine serum albumin, 2mM DTT and 5 μCi/ml ^{3}HdTTP (19 cpm /pmole). Assays were performed at specified temperatures, and terminated as in 2.13.

Activated DNA was prepared by W. F. Wakeling by the method of Holmes (1974).

2.15 Isopycnic caesium chloride density gradient centrifugation of yeast DNA.

The methodology of caesium chloride density gradient centrifugation has been described extensively (Flamm, et al, 1972). Since 2 μm plasmid DNA has the same density (1.699) as nuclear DNA it must be separated using ethidium bromide-caesium chloride gradient centrifugation (Livingston and Klein, 1977). All solutions were sterilized where possible.

2.15.1 Caesium chloride gradients.

Approximately 10^8 washed spheroplasts (2.9) were resuspended in 0.5ml 5mM Tris-HCl pH8.0, 5mM EDTA and lysed by the addition of 50 μl 17.5% sarkosyl NL35. 50 μl 16mg/ml pronase (Calbiochem) which had been predigested at room temperature for 3 hours was added and the mixture incubated at room temperature for 1 hour. The lysate was spun in an

Eppendorf Zentrifuge 3200, and the supernatant removed and made up to 4.0ml. From this, 3.82 ml was added to 4.733g caesium chloride to give a final refractive index of 1.3985, measured on an Abbé refractometer. The formula of Flamm et al, (1972) relating refractive index to initial density was found not to hold when using lysates containing sarkosyl. 5ml of this solution was transferred to a cellulose nitrate Spinco tube (No.302235) and overlayed with light liquid paraffin. Gradients were formed by centrifugation in a Beckman 50Ti rotor at 16°C and 33K rpm for 60 hours, or 40K rpm for 40 hours. Gradients were fractionated from the bottom of the tube by displacement with liquid paraffin via a 3-way tap (Flamm et al, 1972).

2.15.2 Ethidium bromide - caesium chloride gradients.

The method described by Newlon and Fangman (1975) did not give satisfactory separation of plasmid, nuclear and mitochondrial DNA. An alternative method was described by Livingston and Klein (1977), which used a modification of Hirt (1967) procedure.

10^8 spheroplasts were resuspended in 1.5ml 25mH Tris-HCl pH8.0, 10mH EDTA, and this suspension was added dropwise to 1.5ml of the same solution containing 2% SDS. The tube was inverted gently several times to lyse cells with the minimum shearing of nuclear DNA. 0.75ml 5M NaCl was added, and the tube inverted again. This solution was allowed to stand in ice for 16 hours, then the precipitated material was spun out in an MSE 18 centrifuge at 15K rpm for 1 hour at 4°C. This technique was reported to precipitate 80-90% of the nuclear DNA, leaving 95% of the 2μm-plasmid in the supernatant (Livingston and Kupfer, 1977). The volume of the supernatant was adjusted to 3.75ml, and this was added to 4.800g caesium chloride. 0.5ml 10mg/ml ethidium bromide was added and 10mM Tris-HCl pH7.5, 1mM EDTA to make the final weight 10.880g in each tube. Gradients were spun and fractionated as in 2.15.1.

2.16 Purification of plasmid DNA from bacteria.

All solutions and equipment were sterile. 50ml cultures in L-Broth (2.3) were grown overnight at 37°C. Cultures were centrifuged in an MSE 18 at 10K rpm for 5 minutes at 4°C. After thorough draining, each pellet was resuspended in 1ml 25% sucrose, 50mM Tris-HCl pH8.0 at 0°C, then 0.2ml lysozyme solution (8mg/ml in 0.25M Tris-HCl pH8.0) was added and the mixture kept in ice for 5 minutes. 0.4ml 0.25M EDTA pH8.0 was added and the tube kept in ice for 5 minutes, swirling occasionally. Cells were lysed by the addition of 1.6 ml lysis buffer (1% Brij-58, 0.4% sodium deoxycholate, 62.5mM EDTA, 50mM Tris-HCl pH8.0). The lysate was returned to 0°C for 30-45 minutes, then cleared by centrifugation in a Beckman 50Ti rotor at 27K rpm for 30 minutes at 4°C. The volume of the supernatant was adjusted to 3.8ml, 0.3ml 10mg/ml ethidium bromide was added and this mixture was added to 3.550g caesium chloride (for DS581 ColE1 plasmid). Gradient formation was as described in 2.15.

After centrifugation, plasmid DNA was visible under short wave ultra-violet light as a heavy band in the lower half of the gradient. This was removed through a hole punctured in the side of the tube with a sterile 21G needle. The collected plasmid DNA was extracted six times with an equal volume of caesium chloride saturated isopropanol, then dialysed against 500ml 10mM Tris-HCl pH7.5, 1mMEDTA. Plasmid DNA was stored in sterile sealed tubes at 4°C.

2.17 Agarose gel electrophoresis of DNA.

0.8-1.4% horizontal agarose gels (Marine Colloids Inc.; low electroendosmosis) were run in 40mM Tris-base, 20mM sodium acetate, 10mM EDTA adjusted to pH7.7 with acetic acid (gel buffer was kept and a 10x concentrated stock at 4°C). Gels were 15cm x 20cm x 0.5cm, made by pouring the gel (after steaming for 10 minutes) onto a sellotape-sided glass plate. Sample wells were 0.4cm deep, and could hold 45 µl.

Sample preparation. Routinely 20µl of stop mix (7M Ultrapure urea,

50% v/v sucrose, 50mM EDTA, 0.01% Bromophenol blue, pH7.0) was added to 20-30 µl sample containing 0.1-2 µg DNA.

Electrophoresis was carried out at 40 volts overnight. DNA was visualised by staining in 1 µg/ml ethidium bromide for 30 minutes, then destaining in water for 30 minutes, and observing under short wave U-V light.

2.18 Kleinschmidt spreading of DNA.

The method used was that of Davis et al (1971). Hypophase solution (0.25M ammonium acetate, pH7.5) was poured down a heated and cooled stainless steel ramp into a clean 5cm petridish. While the ramp was still wet, 25 µl of a mixture containing 0.5M ammonium acetate, 100 µg/ml cytochrome-C (Type III, Sigma), 0.5-1.0 µg/ml plasmid DNA, 5mM Tris-HCl pH7.5 and 1mM EDTA. The cytochrome-C, attached to the DNA, forms a monolayer on the surface. After 30 seconds, a carbon-coated EM grid was touched onto the surface, 1 grid width from the ramp and held there for 30 seconds. After a further 20 seconds the grid was dried against filter paper and then held in a drop of stain for 30 seconds. The stain was 1 in 100 dilution in 90% ethanol of 5mM uranyl acetate in 50mM HCl, prepared freshly. The grid was dipped into isopentane for 10 seconds, then dried. Stain and DNA concentrations and staining time were adjusted or necessary to give the best result. The grids were examined using a Phillips EM300 electron microscope.

2.19 Autoradiography and Photography.

Fuji Rx medical X-ray film was used for autoradiographs. This was developed for 5 minutes at 20°C using Ilford Phenisol developer, washed in a 1% acetic acid stop, then fixed in Kodak Unifix for 5 minutes, followed by copious washing.

Photography was performed using a 35mm camera and Ilford FP4 film. Photomicroscopy was performed on a Wild Microscope incorporating a Polaroid camera using Polaroid Type 665 positive/negative film.

Photographs of ethidium bromide stained agarose gels under U-V light were obtained using a Polaroid camera with red filter attached, and Type 55 positive/negative film.

CHAPTER THREE

Characterisation of cdc7.4

3.1 INTRODUCTION

Before commencing a major study into the nature of any of the cdc mutations, it was important to establish that the strains in our possession displayed the same phenotypes as those reported in the literature (1.6), and to extend their characterisation.

Particular attention was given to DNA synthesis. Previous work (Hartwell, 1973; Hereford and Hartwell, 1974), had used the incorporation of 6-^{3}H uracil into alkali resistant, acid precipitable material to monitor DNA synthesis, since thymidine labelling is not possible in S.cerevisiae due to the lact of thymidine kinase activity (Grivell and Jackson, 1968). The validity of this method was tested by determining the sensitivity of the radioactively labelled material to DNAase and RNAase, as there is a risk of interference from the 50-fold excess of RNA known to be present in S.cerevisiae (Carter, 1975).

A frequently used method for achieving cell synchrony in S.cerevisiae is the arrest of a-type cells at "Start" using purified α-factor (Hartwell, 1974; 1.2.1). Cells released from this block show a synchronous burst of DNA replication (Hereford and Hartwell, 1974). It was important to establish that this was nuclear DNA replication. Also, the DNA replicated during α-factor arrest and at the cdc7.4 block was characterised. While this work was in progress, Newlon and Fangman (1975) showed that cdc7.4 continued to synthesise mitochondrial DNA at the restrictive temperature, indicating the independence of mitochondrial DNA replication from the CDC7 gene product.

The effect of the cdc7 lesion upon synthesis of RNA, protein and phospholipid was examined at the restrictive and permissive temperatures, because these aspects have not been adequately described in the literature.

Priming by RNA synthesis appears to be a fairly common solution to the problem of de novo initiation of DNA replication (1.5). The

situation in yeast has not been fully explored, though RNA priming of 4S nascent fragments was suggested by the finding of RNA at their 5' ends in an in vitro system (Oertel and Goulian, 1977). An inhibitor was sought to probe the requirement of RNA synthesis for DNA synthesis. Many inhibitors such as actinomycinD, daunomycin, ethidium bromide, α-amanitin and lomofungin, were considered and found to be unsuitable, either because of multiple effects or incomplete inhibition of all types of RNA synthesis, or inability to enter the yeast cell (Schindler and Davies, 1975). However, one apparently suitable antibiotic which had been studied was thiolutin (Jimenez et al, 1973; Khachatourians and Tipper, 1974; Tipper, 1973). This compound, the structure of which is shown in Fig. 9, inhibits yeast RNA polymerases, I, II and III in vitro, and evidence suggests that it may be a specific inhibitor of RNA synthesis in vivo by binding to and inactivating RNA polymerase (Tipper, 1973). Template was found to protect RNA polymerase from inhibition by thiolutin (Tipper, 1973). Experiments were performed to try to determine whether an essential RNA synthetic step was already completed in cells arrested at the cdc7 block (c.f. rifampicin and the dnaA mutation in E.coli; 1.5.3.1).

An enriched nuclear fraction was obtained from gently lysed spheroplasts by sucrose gradient fractionation, and its purity was assessed by chemical and electron microscopic analysis. Such fractions were used for studying nuclear protein profiles by SDS-polyacrylamide gel electrophoresis in an attempt to detect mutant proteins.

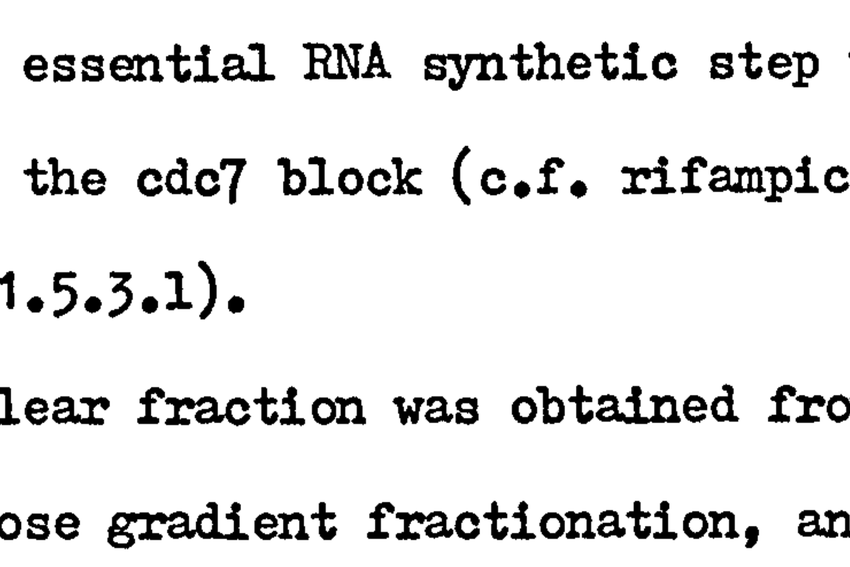


Fig. 9: Structure of thiolutin.
(from Tipper, 1973).

3.2 Methods

3.2.1 Nuclease Digestion.

Samples of culture were treated in 1.3N NaOH as for DNA synthesis determinations (2.6.1). After TCA precipitation and filtration through Whatman GF-C (2.5cm. circles), the filters were dried and transferred to empty scintillation vials, and saturated with 0.2ml digestion buffer (50mM Tris-HCl pH7.8, 8mM $MgCl_2$, 2mM $CaCl_2$). 5µl of 1mg/ml bovine pancreatic DNAaseI (Sigma) in 1mg/ml BSA, or 25µl of 1mg/ml (bovine pancreatic) RNAaseA (Sigma) in 50mM sodium acetate pH5.5 (boiled for 10 minutes) was added as required. Samples were incubated at 37°C overnight. 10ml ice-cold 5% TCA was added and the material from the vials refiltered, dried and counted in Brays scintillation fluid.

3.2.2 Processing of samples for phospholipid/phosphoprotein determinations.

2ml samples of ^{32}P-phosphate pulsed cultures were treated for 1 hour on ice with 0.15ml 98% formic acid (final pH 2.0) before filtering through Whatman GF-C 2.5 cm circles, and washing extensively with ice-cold M formic acid. Filters were air dried for 4-5 minutes then transferred to glass centrifuge tubes and extracted with $CHCl_3$/MeOH (2:1) at 60°C for 5 minutes. Tubes were spun briefly and the extract removed before repeating with $CHCl_3$/MeOH/HCl (124:61:1). Extracts were pooled and evaporated to dryness, the residues being taken up in 0.25ml absolute ethanol. 0.1ml was counted directly in Brays scintillation fluid to give counts present in phospholipid.

Filters remaining after extraction were air dried. Sufficient 5% TCA was added to the filters to cover them, and then the tubes were boiled for 30 minutes. The entire contents of the tubes were collected on further Whatman GF-C circles, and then dried and counted in Brays, to give counts present in phosphoprotein.

The extraction procedure is derived from that of Letters (1968), described in Stewart (1975).

3.2.3 Preparation of yeast nuclei for electron microscopy.

The method used was that of Molenaar et al (1970). All steps were performed at room temperature. The nuclear pellet was fixed in 5% glutaraldehyde in 0.1M sodium cacodylate, pH7.4, for 2 hours, then washed 5 times with 0.2M sucrose in 0.1M sodium cacodylate, pH7.4. After fixing, the pellet was stained for 1 hour in 1% osmic acid in Palade buffer (comprising 5ml 0.14M sodium acetate, 5ml 0.14M sodium barbiturate, 5ml 0.1N HCl and 10ml 2.5% osmium tetroxide, final pH7.4). Finally, the pellet was washed 5 times with physiological saline (0.95%) pH7.4 and dehydrated with alcohol. Embedding in Epon and sectioning was performed by C. Davie.

3.3 Results and Discussion

3.3.1 Temperature sensitivity and mating type of the cdc mutants

Cells from a variety of strains, including A364A, cdc4 (H135.1.1), cdc7.3(18032), cdc7.4 (H201.14.4), and cdc28.1 (H185.3.4), were checked for their terminal phenotypes by growth at 37°C for 3½ hours in YEPD-AU medium. The results of photomicroscopy are shown in Fig.10. The results can be summarised as follows: A364A had cells at all stages of the division cycle present (in an exponential culture, approximately 40% of cells are unbudded); cdc4 gave rise to cells with multiple elongated buds; cdc28 resulted in single unbudded cells, carrying a small probascis ("schmoo") which can be distinguished from an emergent bud; cdc7 strains arrested with the dumb-bell morphology. These results confirm those of Hartwell (1974).

The cdc mutants were shown to be a-mating type by exposure of low concentrations of cells to α-factor present in wells bored into YEPD-TAU plates. Plates were incubated at 23°C for 4-5 hours, and observed microscopically. "Schmoo" forms confirmed a-mating types (data not shown.

3.3.1.1 Cell Viability.

Cell viability estimates (2.5) were performed on exponential cultures of A364A, cdc7.1 (124), cdc7.3 (18031), cdc7.4 (DE200.1.3; 4.3.2.4), and cdc7.7 (E247) growing in YEPD-AU after a shift to 38°C. The results presented in Fig.11 show that the cdc7.4 mutant begins to lose viability after more than 2 hours at 38°C. This confirms the earlier report of Hereford and Hartwell (1974), and demonstrates the necessity of using α-arrest followed by brief exposure ($\sim$ 2 hours) to the restrictive temperature to achieve synchronous arrest at the cdc7.4 block. Other mutants, e.g. cdc4, cdc28.1 can be arrested at the cdc block by exposure to the restrictive temperature for 1 generation (about 3 hours) without loss of viability (J.B. Taylor, pers.

Figure 10: Terminal phenotypes of cells grown at 38°C.

Cells of strains A364A, cdc4 (H135.1.1), cdc28.1 (H185.3.4), cdc7.3 (18032) and cdc7.4 (H201.14.4) were grown at 38°C in YESD-AU_{10} or on solid medium for various times at 38°C. The left hand column shows cells grown on YESD-AU_{10} for 3½ hours. The right hand column shows cells grown on YEPD-AU plates for 8 hours. Note that cdc28 formed obvious "schmoos" when grown on agar plates but not when grown in liquid culture.

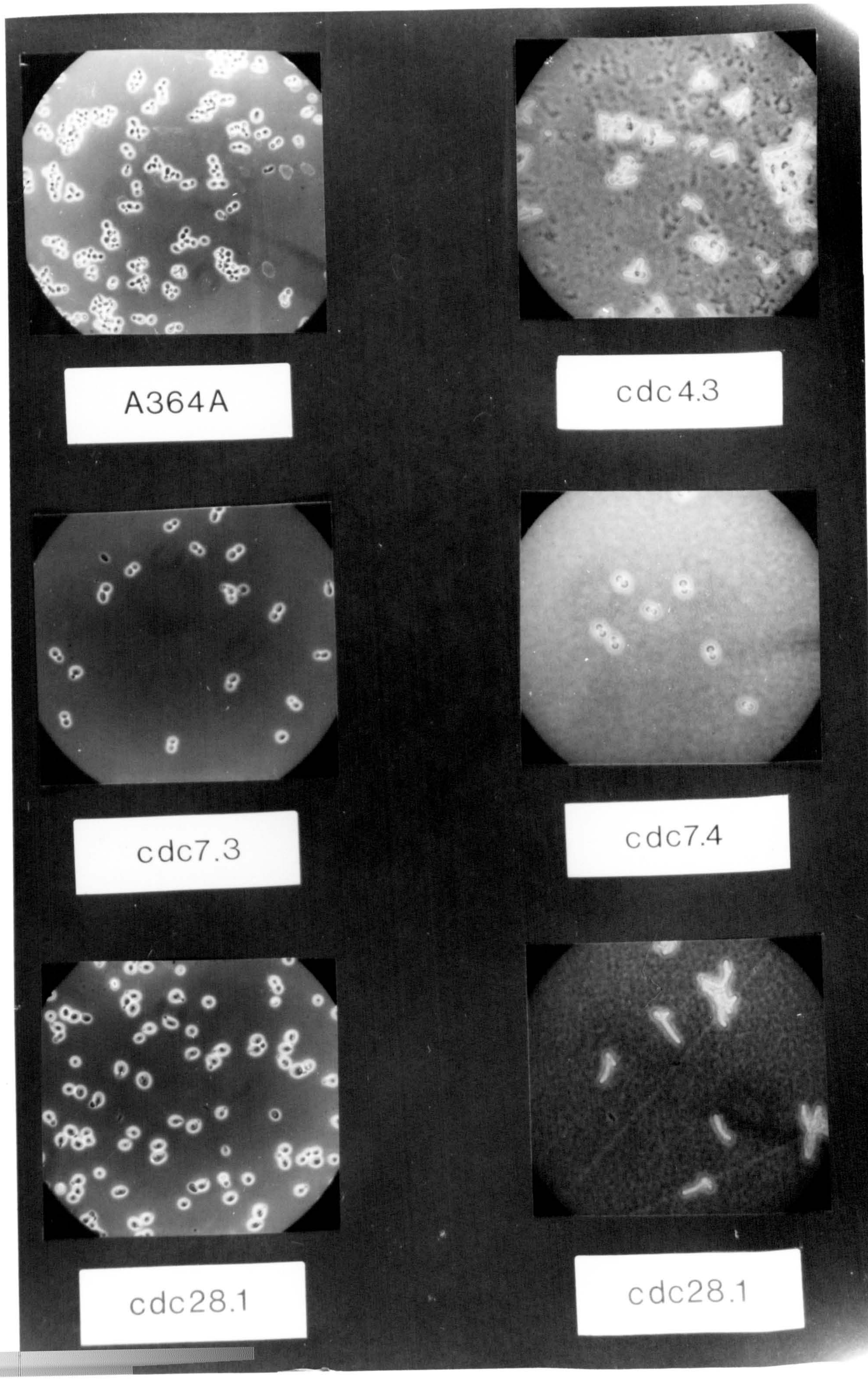
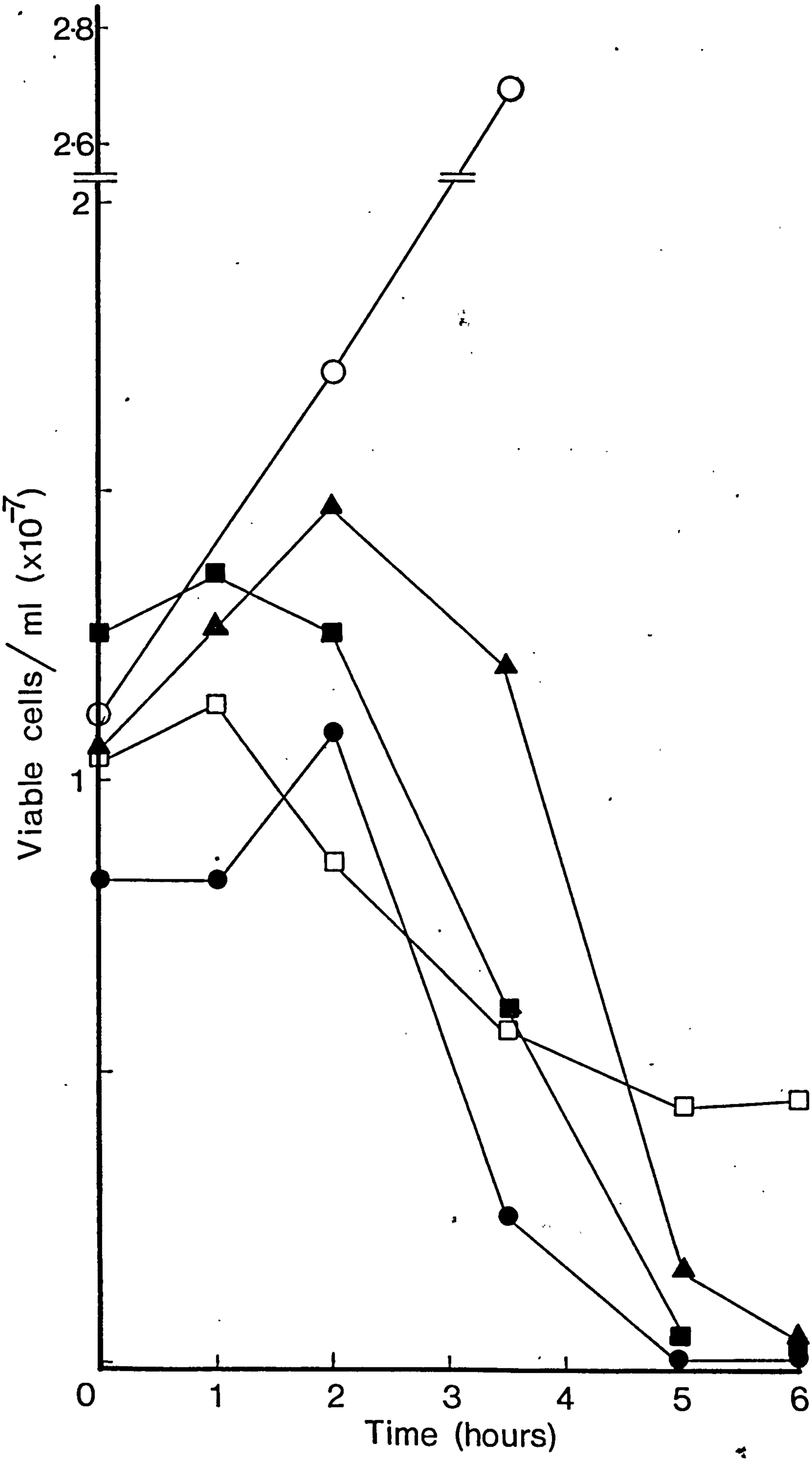

A364A
cdc4.3
cdc7.3
cdc7.4
cdc28.1
cdc28.1

Figure 11: Cell viability at 38°C.

10ml exponential cultures of A364A, cdc7.1 (124), cdc7.3 (18032), cdc7.4 (DE200.1.3) and cdc7.7 (E247) in YEPD-AU medium, were adjusted to 10^7 cells/ml using OD_{600}, and shifted to 38°C. 0.1ml samples were removed at the indicated times and diluted into 0.3ml sterile water then sonicated for 4 seconds with the microprobe on an MSE sonicator (this does not affect overall viability) to disrupt clumps. Two fifty-fold serial dilutions were performed in sterile water, and 0.1ml of the diluted cell suspension was spread on YEPD-AU agar plates until the liquid had dried. Plates were incubated at 23°C for 2 days before scoring. 50 colonies per plate corresponded to 5×10^6 viable cells/ml.


2·8
2·6
2
1
Viable cells / ml (x10⁻⁷)
0
1
2
3
4
5
6
Time (hours)

comm.). The viability of cdc7.7 is markedly better than cdc7.4.

3.3.2 DNA synthesis in cdc7.4

3.3.2.1 Overall pattern of DNA synthesis

DNA synthesis was monitored by incorporation of 6-^{3}H uracil into alkali-resistant, TCA-precipitable counts. The protocol involved synchronisation of the culture with α-factor, followed by a short exposure (105 minutes) to the restrictive temperature to arrest the cells at the cdc7 block. Figure 12 shows the effect of temperature upon DNA synthesis in cdc7.4. The characteristic failure of cycloheximide to inhibit DNA synthesis upon shift down of cultures held at the cdc7 block was reproduced. A pronounced lag of about 45 minutes was observed in cdc7 cultures released from the α-factor block to 23°C. This was greater than that observed in experiments with other cdc mutants (data not shown). However, this lag could be substantially reduced by using centrifugation of cells instead of filtration through Millipore filters as the means of removal of α-factor. The gradual creep up of counts during α-factor arrest and at 38°C is due to mitochondrial DNA replication (3.3.2.2).

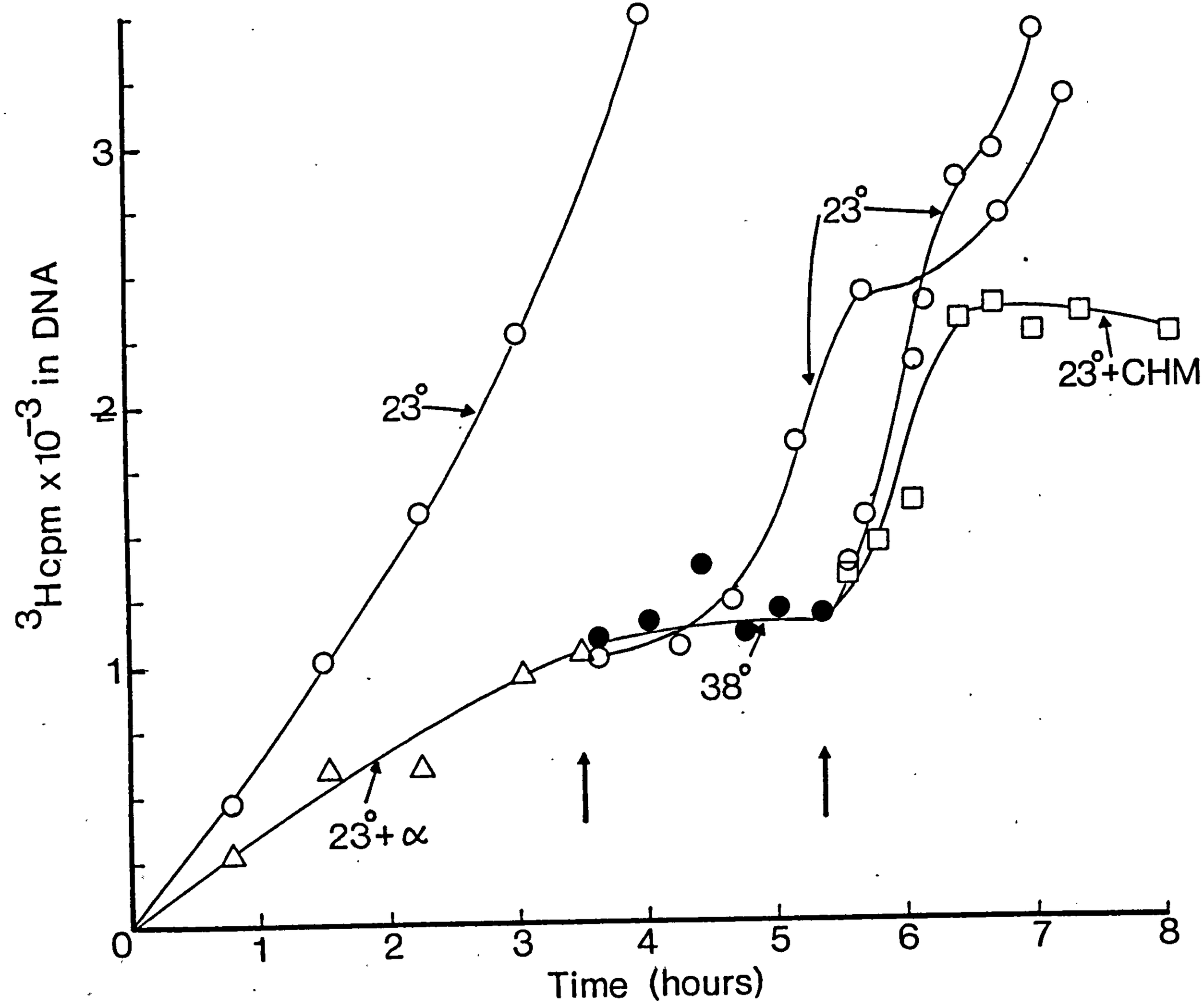
It must be noted that attempts to label DNA in cells grown under the conditions described by Hereford and Hartwell (1974) were unsuccessful. Their medium contained only 0.2% glucose, which appeared to restrict growth to a maximum of $2x10^{7}$ cells/ml. Significant incorporation was not seen at the cell density and specific activity of 6-^{3}H uracil used by these workers ($2x10^{6}$ cells/ml and 35 mCi/mmole). The change to YESD-AU_5 medium and the use of a higher starting cell density and specific activity ($5x10^{6}$ cells/ml and 70 mCi/mmole), overcame this problem.

3.3.2.2 Characterisation of DNA.

The nature of the alkali-resistant, TCA-precipitable material was

Figure 12: DNA synthesis in cdc7.4 (H201.14.4).

A 50ml culture of cdc7.4 (H201.14.4) was grown overnight at 23°C in YESD-AU_5 then the cells were removed by centrifugation in an MSE bench centrifuge and resuspended in the same medium containing 4 μCi/ml 6-^{3}H uracil (70mCi/mmole). Initial cell density was $2.1x10^6$ cells/ml. 0.5ml samples were removed throughout the experiment and processed for radioactive incorporation into DNA as described in 2.6.1. except that samples were incubated overnight at 37°C in alkali. The culture was split into 40ml and 10ml portions, both grown at 23°C; the 40ml portion received 0.4ml α-factor in methanol (2.8), and the 10ml culture received 0.1ml methanol. After 3½ hours the α-factor treated culture was split into 12 ml and 25ml portions which were filtered through Millipore HAWP02500 filters (equilibrated at 23°C and 38°C respectively). Cells were washed with 50ml and 100ml fresh medium at 23°C and 38°C respectively, then resuspended in 12ml and 25ml fresh YESD-AU_5 containing 4 μCi/ml 6-^{3}H uracil at 23°C and 38°C. After 105 minutes at 38°C, the large culture was split into 2x10ml portions, one of which received 0.1ml 10mg/ml cycloheximide, and both were shifted to 23°C.


^{3}Hcpm x 10^{-3} in DNA
3
2
1
0
1
2
3
4
5
6
7
8
Time (hours)
23°
23°+α
38°
23°
23°+CHM

examined in a comparable experiment to that of Fig.12, by filtering the precipitates onto Whatman GF-C circles and treating with DNAaseI, RNAase A or buffer, as described in 3.2.1. Culture samples were removed at 1 hour intervals throughout the experiment for nuclease digestion and cell viability estimation, since it was important to establish that failure to incorporate radioactivity was not caused by cell death. The results of this experiment are shown in Table IV. Nuclease digestion revealed that the alkali resistant counts were on average 89.6% resistant to RNAase, and only 14.1% remained after exposure to DNAase. This confirmed the material as DNA, but suggested a small amount of residual RNA was present. In later experiments, RNA contamination was virtually eliminated by treating all samples at 60°C in 1.3N NaOH for 3 hours prior to processing. Samples removed throughout the experiment were equally susceptible. Cell viability was maintained in cells held at 38°C for up to 2 hours (3.3.1.1), though 100µg/ml cycloheximide decreased the viability of synchronised cells released at 23°C. This may account for the incomplete doubling of DNA in cycloheximide treated cells released from the cdc7 block compared with untreated released cells, seen in Fig.12. The apparent drop in viability after α-factor treatment, without a concomitant drop in labelled DNA was probably due to the clumpiness of the cells after Millipore filtration, which made disruption for plating more difficult. Mating arrest is known to cause cell agglutination (Sakai and Yanagishima, 1971).

In S.cerevisiae, nuclear DNA (ρ = 1.699 g cm^{-3}) can be separated from mitochondrial DNA (ρ = 1.680 g cm^{-3}) by isopycnic caesium chloride density gradient centrifugation (Flamm et al, 1972; Newlon and Fangman, 1975). This technique was used to determine the nature of the DNA synthesised during and after release from α-factor arrest. Fig.13a shows that DNA labelled with 6-^{3}H uracil during the last 1.5 hours of a 4.5 hour α-factor treatment was predominantly mitochondrial, as

TABLE IV Nuclease sensitivity of alkali resistant TCA precipitable radioactivity.

SAMPLE	Alkali resistant TCA precipitable cpm.	% cpm remaining after digestion with RNAse	DNAse	Viable cells/ml (x10-7)
Time zero	-	-	-	1.0
1 hour + α-factor 23°C	330	84.2	5.2	1.1
2 hours + α-factor 23°C	350	111.9	11.9	1.2
3½hours + α-factor 23°C	605	81.0	34.2	1.4
α-factor removed after 3½ hours, culture split and resuspended at 23°C and 38°C.				
0 hour, 23°C	547	73.1	36.6	1.1
1 hour, 23°C	773	93.8	3.5	1.0
2 hours, 23°C	1373	82.7	2.8	1.0
3 hours, 23°C	1334	97.5	13.8	1.8
0 hour, 38°C	618	93.9	16.3	1.0
1 hour, 38°C	673	82.9	8.3	1.1
2 hours, 38°C	724	86.9	12.6	1.1
After 2 hours at 38°C, culture split, one part shifted to 23°C, the remainder shifted to 23°C + 100 µg/ml cycloheximide (CHM).				
0 hour, 23°C	1387	106.7	17.3	1.1
1 hour, 23°C	1744	92.9	11.3	1.4
2 hours, 23°C	2086	86.3	7.2	1.6
0 hour, 23°C + 100µg/ml CHM	1064	82.2	7.8	1.1
1 hour, 23°C + 100µg/ml CHM	1373	95.2	14.8	1.3
2 hours, 23°C + 100µg/ml CHM	1676	87.3	5.8	1.0
		Average 89.6%	Average 14.6%	

judged from the correspondence with ^{14}C-labelled DNA derived from exponentially grown A364A cells. Upon release from mating arrest, the DNA synthesised shows the normal class distribution, about 10-20% being mitochondrial (Fig.13b). This was an important point to establish, since mitochondrial DNA replication can give rise to artefactual results. Hereford and Hartwell (1971) reported an <u>in vitro</u> DNA synthesising system employing toluene-treated yeast cells. However, they failed to characterise the product of synthesis, which was shown by Banks (1973) to be exclusively mitochondrial DNA.

A similar study showed that DNA labelled at 38°C with 6-^{3}H uracil for 2.25 hours after 1.5 hours preincubation at 38°C was mainly mitochondrial in cdc7.4 (Fig.13c). Similar results were obtained for cdc4 and cdc28 (data not shown).

DNA synthesised in the presence of cycloheximide upon release of a cdc4 culture from its temperature block has been shown to be mostly nuclear (Hereford and Hartwell, 1973).

3.3.3 RNA and protein synthesis in cdc7.4

Protein synthesis was measured by incorporation of ^{14}C amino acid mixtures (asp,ser,leu,phe,lys) or ^{14}C-protein hydrolysate (both Radiochemical Centre, Amersham), into precipitable counts resistant to boiling in TCA (Hartwell, 1967). A corresponding protocol to that of Fig.12 was used, and the result is shown in Fig.14. The most obvious point is that the cdc7 lesion does not affect amino acid incorporation at 38°C. The original work of Hartwell (1967) used a single 3 hour time point, thus no information could be gained about the rate of protein synthesis because his results could be explained by rapid incorporation and subsequent loss. These data show that the rate of protein synthesis at 38°C is faster than at 23°C, and that a culture shifted from 38°C to 23°C reverts to the typical 23°C rate after a lag phase lasting about 45 minutes. This observation was confirmed by later

Figure 13: Nature of DNA synthesis during and after α-factor arrest, and at 38°C.

A 25ml culture of cdc7.4 (H201.14.4) was grown overnight at 23°C to $7.3x10^6$ cells/ml in YESD-AU_{10}. 0.6ml α-factor (2.8) was added and growth was continued for 3 hours. The culture was split into 2x12ml portions, one of which received 6 μCi/ml 6-^{3}H uracil, and growth was continued for 1.5 hours. The radiolabelled culture was then processed as described in 2.15.1, and the result is shown in Figure 13a. The unlabelled culture was washed free of α-factor by filtration through Millipore HAWP02500 followed by 50ml YESD-AU_{10}, then resuspended in 15ml fresh YESD-AU_{10} containing 6 μCi/ml 6-^{3}H uracil and grown for 2 hours at 23°C. This culture was processed for Figure 13b. To each culture sample prior to processing was added 5ml of A364A culture grown for at least 5 generations in the presence of 0.125 μCi/ml 2-^{14}C uracil in YESD-AU_5 at 23°C.

Figure 13c shows the result for a 10ml culture at $9x10^6$ cells/ml grown in YESD-AU_{10} at 38°C for 1¾ hours then labelled for 2 hours at 38°C with 10 μCi/ml 6-^{3}H uracil. A ^{14}C marker was added as above.

Refractive indices were measured on an Abbe refractometer. The relationship with density used by Flamm et al (1972) did not hold for caesium chloride gradients containing sarkosyl. The designations nuclear (n) and mitochondrial (m) were therefore based on refractive index difference.

a.

b.

c.

n

m

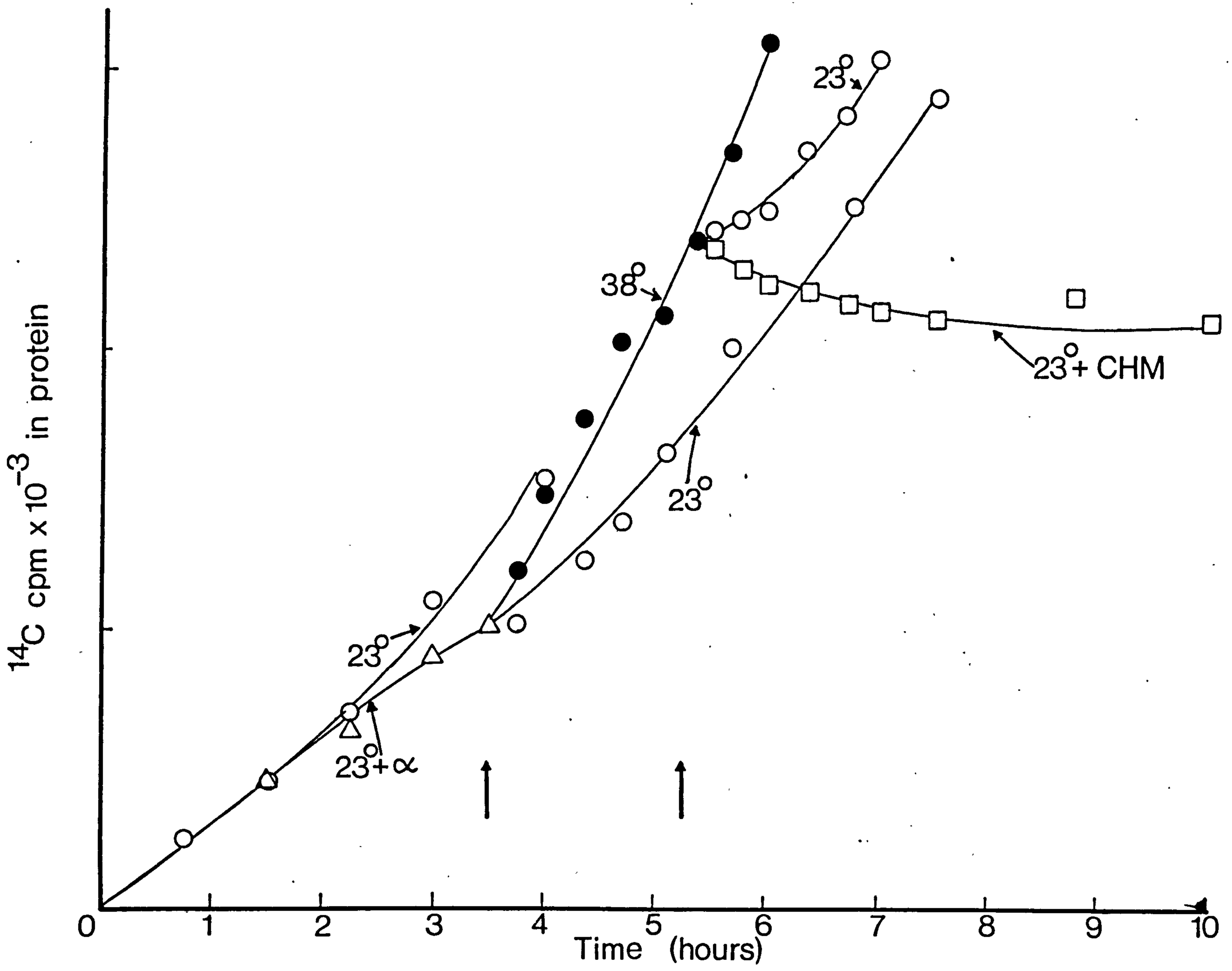
^{14}Ccpm x10^{-2} in DNA

^{3}Hcpm x10^{-3} in DNA

Fraction number

Figure 14: Protein synthesis in cdc7.4 (H201.14.4)

A 50 ml culture of cdc7.4 in YESD-AU_{10} was treated in exactly the same way as described in Figure 12, except that 6-^{3}H uracil was replaced by 0.2 μCi/ml (U-^{14}C) protein hydrolysate and 0.5ml samples were removed at the indicated times and processed as described in 2.6.3 for radioactive incorporation into protein.

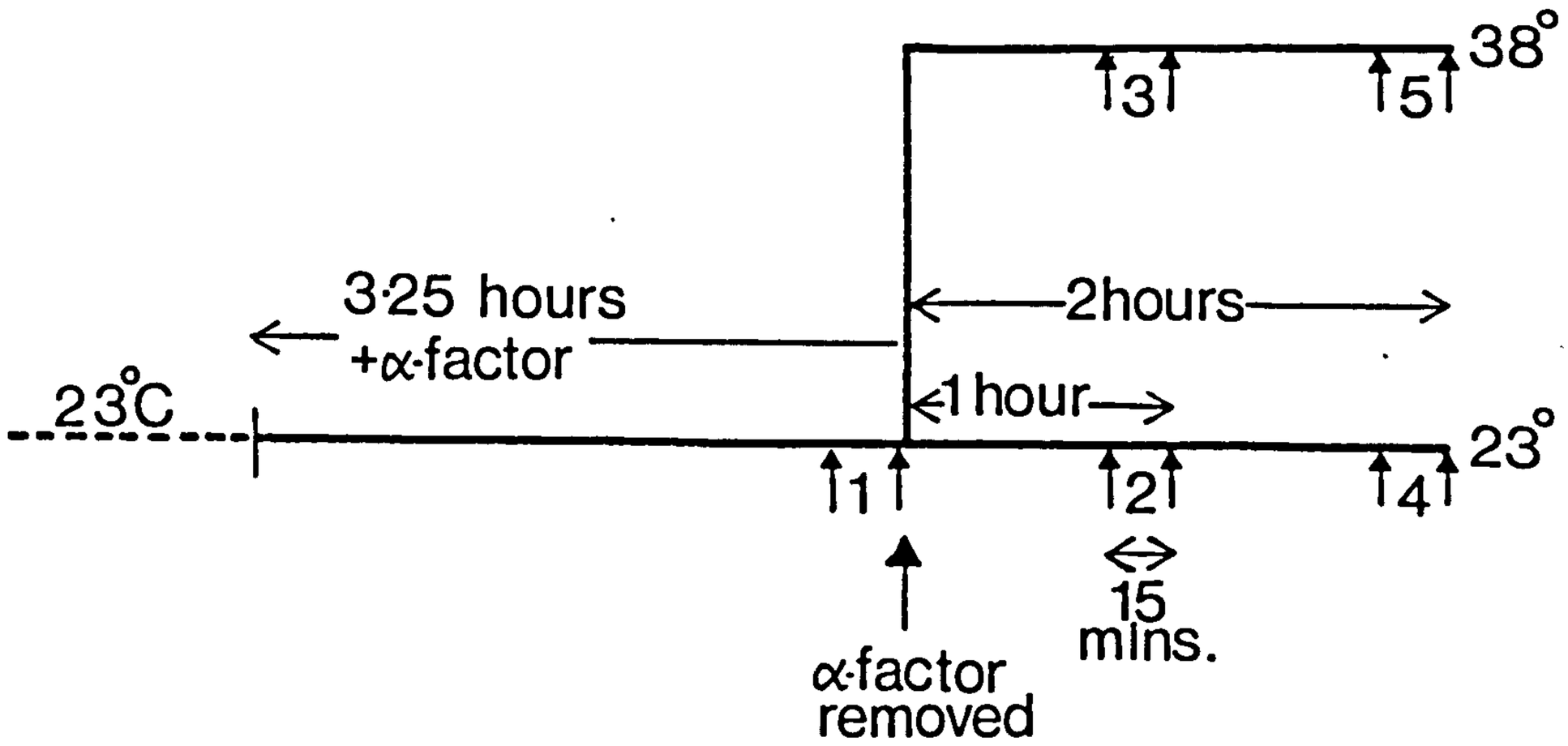

^{14}C cpm x 10^{-3} in protein
38°
23°
23°
23°+ CHM
23°
23°+ α
0
1
2
3
4
5
6
7
8
9
10
Time (hours)

protein labelling studies, and is not strain specific (4.3.1.1). Cycloheximide at 100 μg/ml not only inhibits protein synthesis, but also causes a slight loss of prelabel, which may be associated with the viability loss reported earlier (3.3.2.2). α-factor appeared to depress protein synthesis after 2 hours, due undoubtedly to its effect on cell multiplication. Time course experiments were not performed on A364A because gross incorporation was found to be similar to the mutants.

RNA synthesis was measured by incorporation of 6-^{3}H uracil into TCA precipitable material. This actually monitors total nucleic acid, but since DNA contributes only about 2% (Carter, 1975) this was ignored, The rates of RNA synthesis in cdc7.4 at 23°C and 38°C are indistinguishable from the corresponding rates in A364A over a 5 hour period, confirming earlier reports (Hartwell, 1967), (data not shown).

These data do not exclude the possibility that a mutant may be defective in the transcription of a specific message, or the translation of a specific message into proteins. Examples of the former are not known, but there are a growing number of cases of specific translation defects, presumably through mutation of recognition sites. One such is the rII B cistron of bacteriophage T4, a temperature sensitive mutant of which was shown to be defective in translation (Singer and Gold, 1976). Later work showed that the ts lesion was caused by an alteration in the AUG codon at the start of the coding sequence (Belin et al, 1979). Landy-Otsuka and Scheffler (1978) showed that a ts cell cycle mutant of Chinese Hamster cells was defective in the translation of m.RNA which results in lack of inducibility of ornithine decarboxylase at the restrictive temperature. However, the failure of cycloheximide to block DNA synthesis in cultures released from the cdc7 block proves that de novo protein synthesis is not required, thus the problem in cdc7.4 is one of protein conformation.

Figure 15: Protocol for ^{32}P-phosphate labelling of phospholipids and phosphoproteins.



15ml cultures of A364A and cdc7.4 were grown overnight in low phosphate (low Pi) medium (Rubin, 1973). This medium consisted of YEPD-AU (2.3) depleted of inorganic phosphate by the addition of 10ml 1M $MgSO_4$ and 10ml concentrated ammonia solution to 1 litre YEPD-AU, and after standing for 30 minutes, removal of the precipitate by filtration through Whatman No.1 filter paper. The pH was adjusted to 5.8 before autoclaving.

Each 15ml culture was subcultured to approximately 10^7 cells/ml at the start of the experiment. 0.15ml α-factor in methanol was added to each culture, and growth continued at 23°C. After 3.25 hours, α-factor was removed by filtering the cells through Millipore HAWP02500 and washing with 50ml fresh low Pi medium at 38°C. Cultures were resuspended in 13ml fresh low Pi medium at 38°C and split into two 6.5ml portions. One portion remained at 38°C, the other was shifted to 23°C. At the times shown, marked 1, 2, 3, 4 and 5, 2ml samples were removed and pulsed with 50 μCi/ml ^{32}P-orthophosphate (2.1) for 15 minutes. Pulsed samples were processed as described in 3.2.2. to separate phospholipid and phosphoprotein.

3.3.4 Phospholipid, phosphoprotein and phosphorylated nucleotide syntheses.

The fact that cdc7.4 cannot tolerate exposure to the restrictive temperature for long periods (3.3.1.1), whereas mutants such as cdc28 will regrow at 23°C after several days at 37°C (unpublished observations), suggests that the lesion in cdc7.4, if it is responsible for this effect, may have more damaging consequences than an inability to initiate DNA replication. It was, however, not possible to conduct an exhaustive survey of putative metabolic defects. Certain of them will be referred to in Chapter 5 (5.3.1).

Greengard (1978) reviewed the evidence suggesting that many neurotransmitters, hormones and other regulatory agents achieve their biological effects by altering the phosphorylation of specific proteins. The rapid commencement of DNA synthesis upon release from the cdc7 block is consistent with the lesion affecting a regulatory event such as protein phosphorylation. Incorporation of ^{32}P-phosphate into phospholipid, phosphoprotein and nucleotides can be determined fairly easily, and was relevant to a general characterisation of the yeast strains.

3.3.4.1 Phospholipid and phosphoprotein estimations were performed on the same set of samples using the familiar protocol of arresting cells with α-factor for 3.25 hours, then releasing from the block to either 23°C or 38°C, as shown in Figure 15. Experiments were performed on A364A and cdc7.4 (H201.14.4). At specific times (3 hours in α-factor, 1 and 2 hours after release) 2ml samples were removed from the master cultures and pulsed for 15 minutes to high specific activity with carrier free ^{32}P-phosphate. Low molecular weight compounds were removed by formic acid extraction. Phospholipids were extracted using a $CHCl_3$/MeOH/HCl method (Letters, 1968; Stewart, 1975), and remaining counts (phosphoprotein) precipitated with 5% TCA. A rich medium depleted of inorganic phosphate (low Pi) was used in these experiments (Rubin, 1973).

TABLE V. Phospholipid Synthesis in cdc7.4 (H201.14.4) and A364A.

^{32}P cpm in PHOSPHOLIPID

Expt	Strain	SAMPLES				
		1 α-factor	2 1 hour, 23°C	3 1 hour, 38°C	4 2 hours, 23°C	5 2 hours, 38°C
I	A364A	146,600 (1.00)	277,600 (1.90)	132,600 (0.91)	163,700 (1.12)	252,600 (1.73)
	cdc7.4	243,900 (1.00)	105,900 (0.43)	67,500 (0.28)	85,000 (0.35)	118,900 (0.49)
II	A364A	6,380 (1.00)	10,160 (1.59)	4,100 (0.63)	13,940 (2.18)	15,160 (2.38)
	cdc7.4	41,500 (1.00)	21,260 (0.51)	28,270 (0.68)	22,290 (0.54)	37,840 (0.91)

Notes: Samples 1, 2, 3, 4 and 5 correspond to those described in Figure 15.

Figures in parentheses relate counts in each sample to sample 1

The results of two experiments on phospholipid synthesis are presented in Table V. Two points can be made :-

(1) Phospholipid synthesis never reached its α-blocked value in cdc7.4 even 2 hours after removal of α-factor, at either 23°C or 38°C, whereas A364A rapidly increased synthesis after α-factor removal at both temperatures.

(2) 38°C does not appear to exert any greater inhibitory effect than does 23°C in cdc7.4. In fact, pulse values for cdc7.4 2 hours after α-factor removal are higher at 38°C than at 23°C in both experiments.

The data suggest that phospholipid synthesis in cdc7.4 during recovery from α-factor arrest is different to that in A364A. However, this effect does not appear to be temperature-sensitive. It may be caused by a further defect in cdc7.4, or it may be a side effect of the lesion in DNA replication, through an effect on some precursor common to both phospholipid and DNA synthesis (e.g. CDP, SAM). The roles of SAM (involved in phospholipid synthesis) and mevalonic acid (involved in cholesterol biosynthesis) in DNA replication will be discussed further in Chapter 5.

The corresponding phosphoprotein data from these and other experiments have not been included here. In seven experiments, no temperature-sensitive difference in gross phosphoprotein labelling was observed. In experiments using centrifugation as the means of removal of α-factor, the incorporation in both A364A and cdc7.4 at 23°C and 38°C was lower than in corresponding experiments using filtration. These effects may be attributable to the degree of "stickiness" of the cells observed during and after α-factor release (3.3.2.2), which may cause problems of accessibility and recovery, dependent on the technique used.

Confirmation that the effect on phospholipid synthesis is due to the cdc7.4 mutation could be obtained by performing the experiment

in Fig.15 on outcrossed strains to establish that cosegregation always occurs. In later work, an outcrossed strain of cdc7.4 referred to as DE200.1.3, which was known to lack two additional defects carried by its parent H201.14.4 (4.3.2.4), was tested for the effect of recovery from α-factor arrest on phospholipid synthesis. When compared with A364A, this strain showed no decrease in labelling of phospholipid or phosphoprotein after release from the α-block (data not shown). This suggests that the effect observed in Table V is not caused by the defect in DNA synthesis.

3.3.4.2 Phosphorylated nucleotides were separated by 2 dimensional ascending chromatography on polyethyleneimine cellulose plates by the method of Randerath, (1964). The method was not sensitive enough to detect deoxynucleotide triphosphates in the formic acid extracts from pulsed cells. Reichard (1978) dismussed pool sizes of dNTPs in polyoma infected mouse fibroblasts, and concluded that they were small, particularly that of dGTP. The method of Skoog (1970) is reputedly more sensitive, and may be more suitable for estimations using S.cerevisiae.

However, it can be concluded indirectly that the pools of dNTPs are not affected in cdc7.4. Figures 13a and 13c show that mitochondrial DNA continues to be labelled during α-factor arrest, and at 38°C. Mitochondrial DNA replication uses the cellular pool of dTTP, since cdc21, defective in thymidylate synthetase (Game, 1976) arrests all types of DNA replication immediately upon shift up to the restrictive temperature. Thus, adequate pools of dNTPs must exist in cdc7.4 at 38°C.

3.3.5 Purification of nuclei from the cdc mutants.

Nuclei from lysed or disrupted suspensions of yeast cells have been purified by centrifugation on discontinuous sucrose gradients (Sillevis-Smitt et al, 1972; Groner and Phillips, 1975). This section

describes the successful purification of nuclei from cdc7.4, and the problems that were encountered.

3.3.5.1 Nuclear purification procedure.

A method was sought which would give intact nuclei in high yield. An initial problem was the choice of method for cell disruption. Disruption by glass beads in a Braun homogenizer is suitable for making protein extracts (2.12), but results in extensively sheared DNA, i.e. nuclear disintegration (I.R. Johnston, pers. comm.). Similarly, methods which use pressure disruption, e.g. in a French press (Bhargava and Halvorson, 1971) have been reported to give variable yields (Wintersberger et al, 1973), and almost certainly results in some nuclear disintegration. Thus a method using lysis of spheroplasts was chosen (Groner and Phillips, 1975) since this was reported to give a high yield of nuclei. A variety of techniques were subsequently reviewed by Duffus (1975).

One problem faced was the strain dependent variability in susceptibility to attack by β-glucuronidase (snail-gut juice) a preparation which breaks down yeast cell walls (review, Kuo and Yamamoto, 1975). Figure 16 shows the time course of spheroplast production for cdc28.1 and cdc7.4 as measured by residual light scattering at 660nm after lysis in PNM buffer, pH6.5.

To improve the poor response of cdc7.4 to β-glucuronidase, a pre-treatment with β-mercaptoethanol, as used by Newlon and Fangman (1976) was tried. This did not improve the rate of spheroplast formation (data not shown). Neither did the inclusion of β-mercaptoethanol in the digest. 10mM $MgCl_2$ or $CaCl_2$ was found to be inhibitory, but 5mM EDTA did not cause any stimulation. Zinker and Warner (1976) have also commented on the differences in spheroplasting ability between A364A and its derivatives. cdc7.4 is much less susceptible to snail-gut juice than cdc28.1 and A364A; cdc4 is

Figure 16: Rates of spheroplast formation of cdc28.1 and cdc7.4

10ml cultures of cdc28.1 (H185.3.4,) and cdc7.4 (H201.14.4) were grown overnight in YESD-AU_{10} then diluted to 10^7 cells/ml by OD_{660}nm measurement. Growth was continued for 3 hours before harvesting the cells by centrifugation in an MSE bench centrifuge. Cells were washed once in double-distilled water then resuspended in 2% β-glucuronidase in 1M sorbitol at a concentration of 10^8 cells/ml. At indicated intervals, 0.1ml samples were removed into 0.9ml PMN lysis buffer (10mM PIPES, pH6.5; 0.5mM $MgCl_2$; 10mM NaCl; 1mM DTT: 0.1% spermidine: Groner and Phillips, 1975) plus 1% nonidet. Samples were chilled in ice for 15 minutes before reading OD_{660}nm.

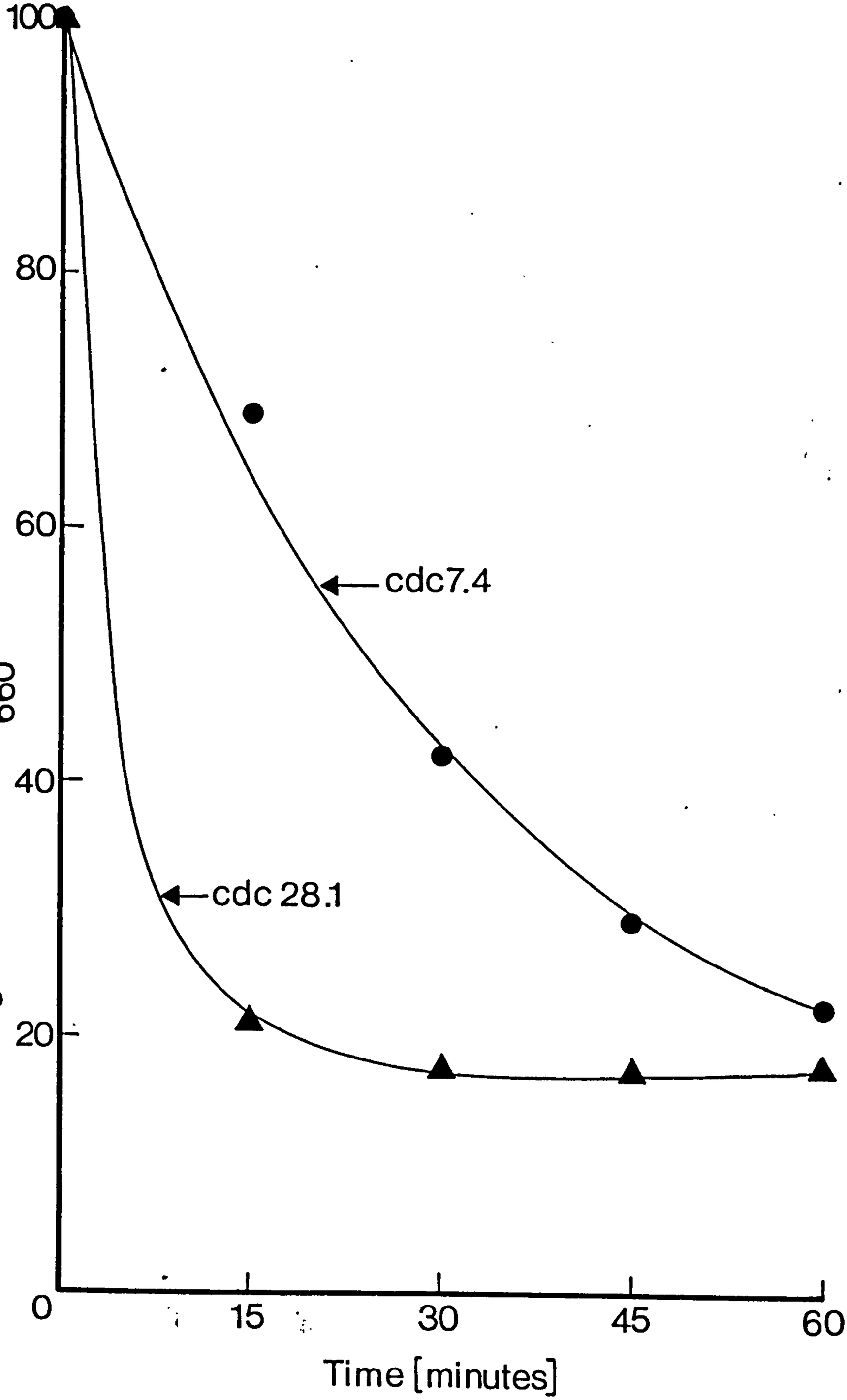

100
80
60
40
20
0
Percentage initial OD660 nm
cdc7.4
cdc 28.1
15
30
45
60
Time [minutes]

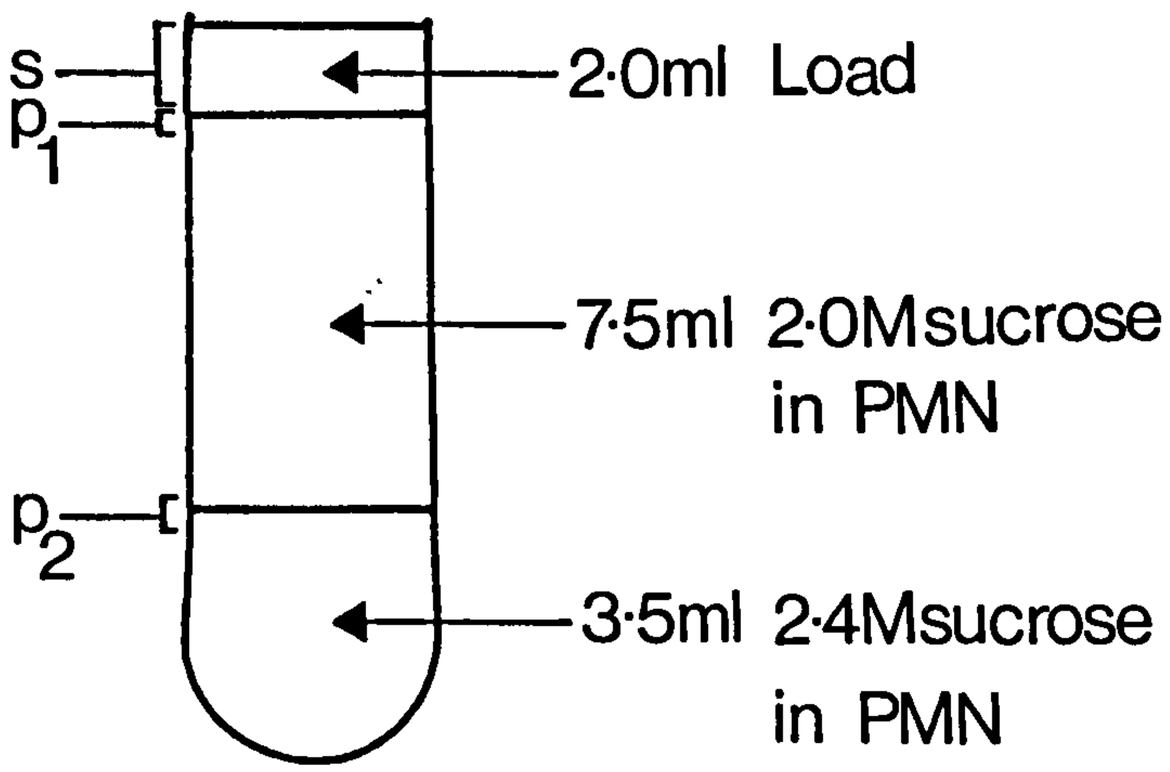
Figure 17: Sucrose gradient technique for preparation of nuclei.

Log-phase cdc7.4 cells were spheroplasted for 45 minutes as described in the legend to Figure 16. Spheroplasts were collected by centrifugation at 3300 rpm for 5 minutes at 4°C in an MSE swing-out bench centrifuge; then washed once in 1M-sorbitol + 5mM EDTA. The spheroplasts were resuspended in 2ml PMN buffer (see legend to Figure 16), containing 1M sorbitol, 1mM $MgCl_2$ and 1mM PMSF. Spheroplasts were lysed gently by the addition of 0.22ml PMN buffer containing 10% nonidet IE. 2ml of this lysate were loaded onto the 11ml stepped gradients shown in the figure, which were centrifuged in a Beckman SW40 rotor at 35K rpm for 15 minutes at 4°C. Samples were removed as shown in the figure using a hooked Pasteur pipette.

intermediate. It was also noted that spheroplasts prepared by this method were not lysed by osmotic shock (i.e. dilution in water).

In later work, an enzyme prepared from the culture fluid of Arthrobacter luteus grown on autoclaved yeast cells (2.9.2) proved much more effective at removing cell walls. Under the microscope, spheroplasts made using β-glucuronidase were refractory (see whole cells in Fig. 10), and often retained their buds, whereas Arthrobacter-produced spheroplasts were rounded and non-refractory, and much more susceptible to osmotic lysis.

The lysate preparation and sucrose gradient centrifugation followed the method of Groner and Phillips (1975), with the addition of a 2.4M sucrose shelf at the bottom of the gradient. This was necessary because a proportion of the spheroplasts always remained unlysed, and pelleted at the bottom of the tube. Fractions were removed from these gradients as shown in Figure 17.



3.3.5.2. Characterisation of Nuclear fractions.

Sucrose gradient fractions were analysed chemically for their content of DNA, RNA and protein (2.7), and also for the recovery of radioactive label in DNA and protein (2.6). The results for cdc7.4 are shown in Table VI. The fraction designated p_2 clearly contained

TABLE VI. Subcellular fractionation of cdc7.4 and comparison with reported nuclear preparations.

STRAIN	Sample	% recovered radioactivity		% recovered macromolecules[1] (chemical estimates)		
		DNA	protein	DNA	RNA	protein
cdc7.4[2]	S	12.7	82.8	10.8(1)	81.0(890)	89.3(3870)
	P_1	18.5	9.2	26.4(1)	14.4(65)	7.8(138)
	P_2	68.8	8.0	62.8(1)	4.7(8.8)	2.9(21.8)
S.cerevisiae Y55[3]	cell	-	-	(1)	(82)	(189)
	nucleus	-	-	(1)	(2.6)	(15.6)
S.cerevisiae s288c[4]	cytoplasm	14.0	92.5	-	-	-
	nucleus	86.0	7.5	-	-	-

Notes: 1) Chemical estimates of macromolecules give the amounts (in μg) expressed as a percentage of the total recovered from each gradient. Figures in parentheses give amounts (in μg) relative to DNA.

2) Similar data were also obtained for cdc4.3 and cdc28.1.

3) Data of Duffus, 1975.

4) Data of Groner and Phillips, 1975.

the bulk of the recovered DNA. Figures in parentheses relate the total amounts (in μg) of RNA and protein to DNA. Corresponding figures from other preparations, reported by Duffus (1975) and Groner and Phillips (1975) are also included in Table VI for comparison.

A p_2 sample from cdc7.4 grown at 23°C was prepared for electron microscopy by the method of Molenaar et al (1970). Fig.18a confirms that the preparation is composed of nuclei, with characteristic "dense crescent" regions which correspond to the nucleoli of higher eukaryotes (Sillevis-Smith et al, 1972). However, this photograph shows that the nuclear membrane was completely removed in the purification. The nuclear structure remains intact. Fig.18b is an enlargement of one nucleus showing an intact mitotic spindle and a spindle plaque.

Extensive work by J.B. Taylor failed to improve the quality of this nuclear preparation, for the cdc mutants. Treatments resulting in the nuclear membrane remaining intact also retained substantial attached cytoplasmic material. The presence of spermidine in the lysis buffer potentiates this effect (J.B. Taylor pers. comm.)

3.3.5.3. Analysis of nuclei on SDS-polyacrylamide gels.

A similar method of separation involved centrifugation of the lysate through a 1.5M to 2.4M sucrose gradient in a Beckman SW50.1 rotor at 20K rpm for 15 minutes at 4°C. Fractions were removed from the bottom of the tube, precipitated with 70% absolute ethanol, and prepared in Studier sample buffer for electrophoresis on SDS-polyacrylamide gels. (2.10). A lysate was prepared from a culture of cdc7.4 grown at 23°C in the presence of 6-^{3}H uracil and (U-^{14}C) protein hydrolysate, to label DNA and protein respectively, and centrifuged through a linear sucrose gradient. Figures 19a and 19b show the ^{3}H and ^{14}C profile of the gradient, and an SDS-polyacrylamide gel of the corresponding fractions. It is interesting to note the gradual changes in several bands (arrowed), as the gradient is descended, identifying proteins that are cytoplasm or

Figure 18: Electron micrographs of cdc7.4 p_2 fraction.

A p_2 fraction from 100ml cdc7.4 grown at 23°C in YESD-AU_{10} medium to a cell density of $9x10^6$ cells/ml was prepared as described in 3.3.5.1. The sample was fixed and stained for electron microscopy as described in 3.2.3. Figure 18 shows a complete field (magnification x8000). Figure 18b is a further magnification (x 41,000) of the mitotic spindle seen in Figure 18a. (Abbreviations; sp, spindle plaque; dc, dense crescent).

Fig. 18a

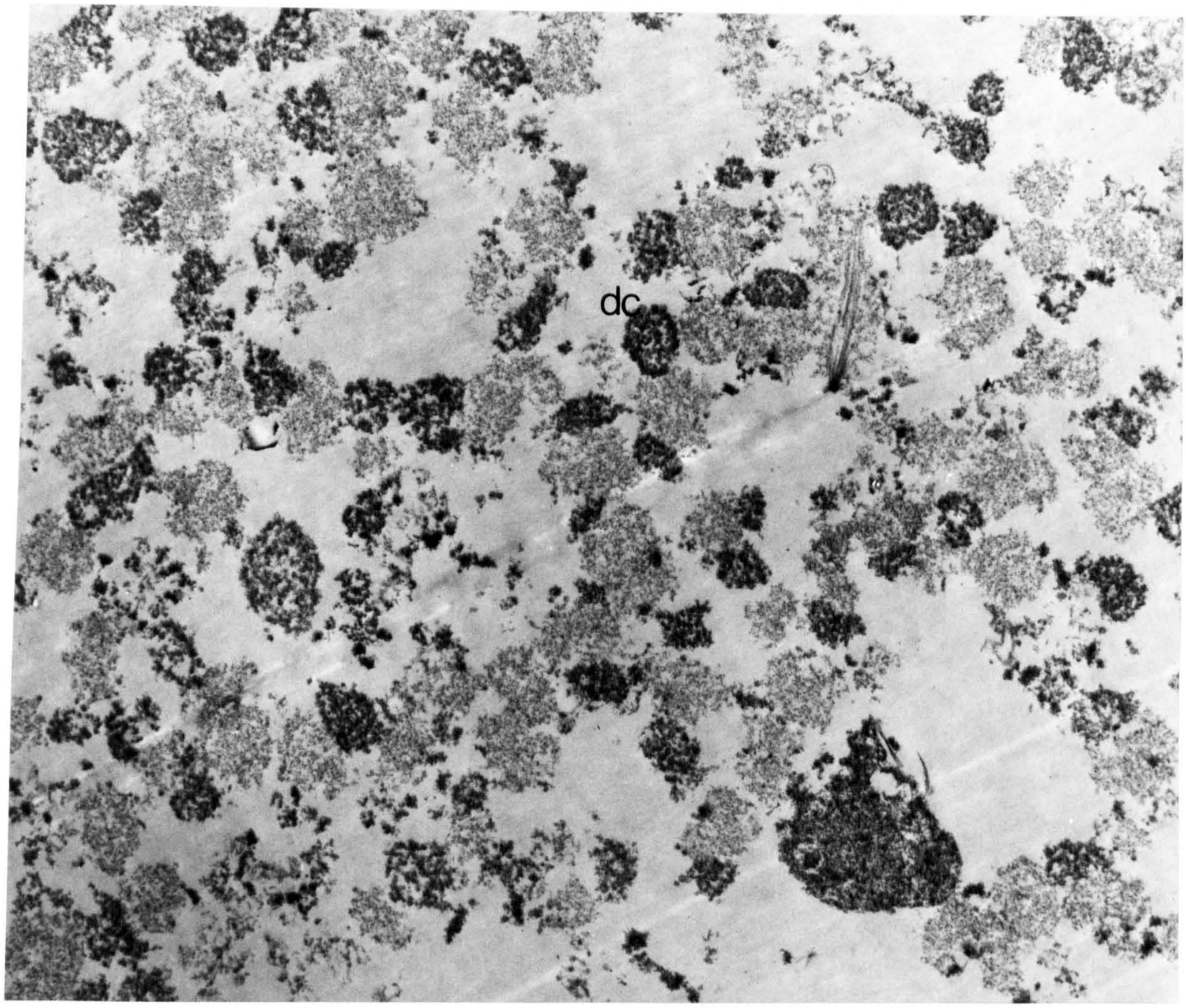


Fig. 18b

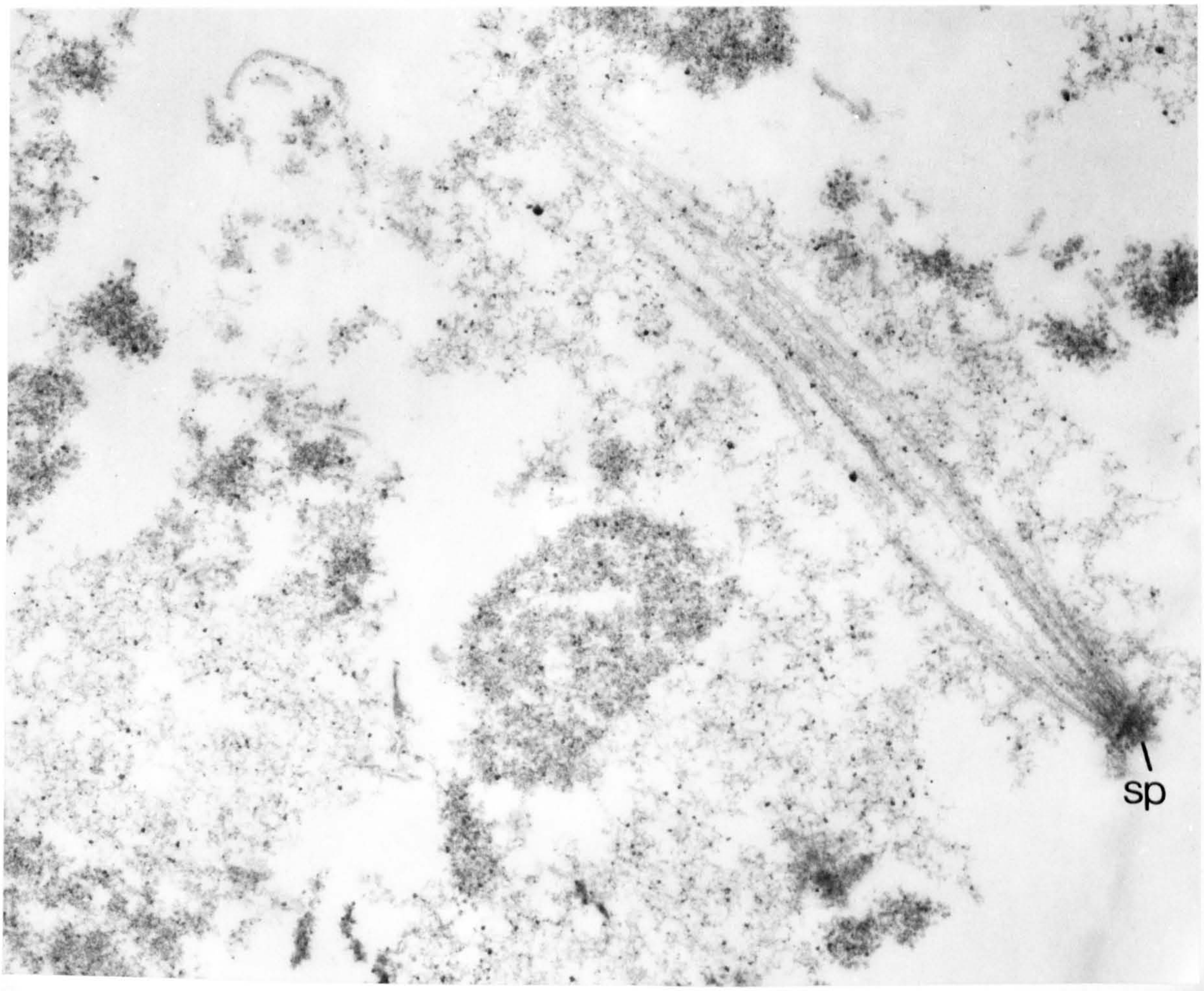
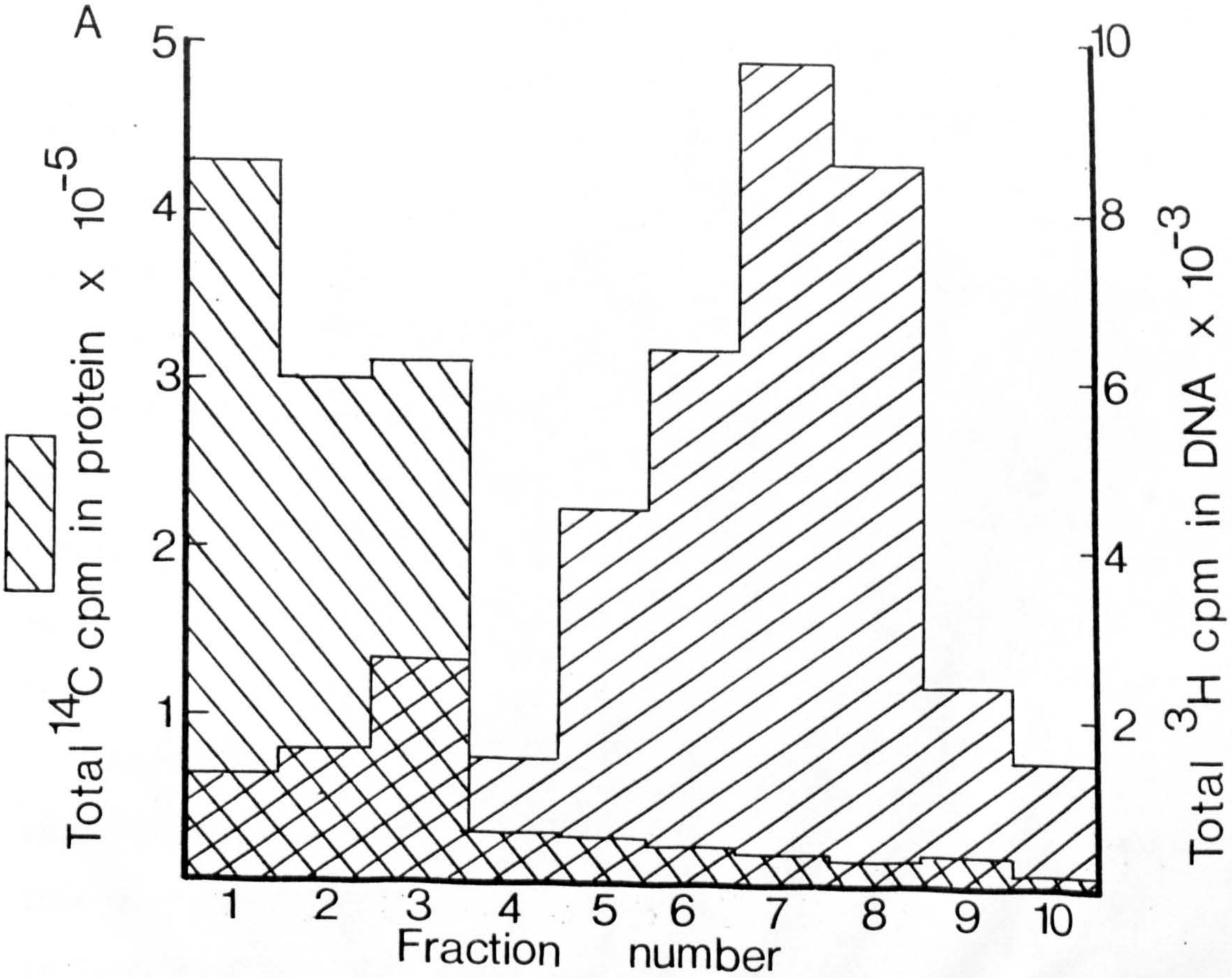


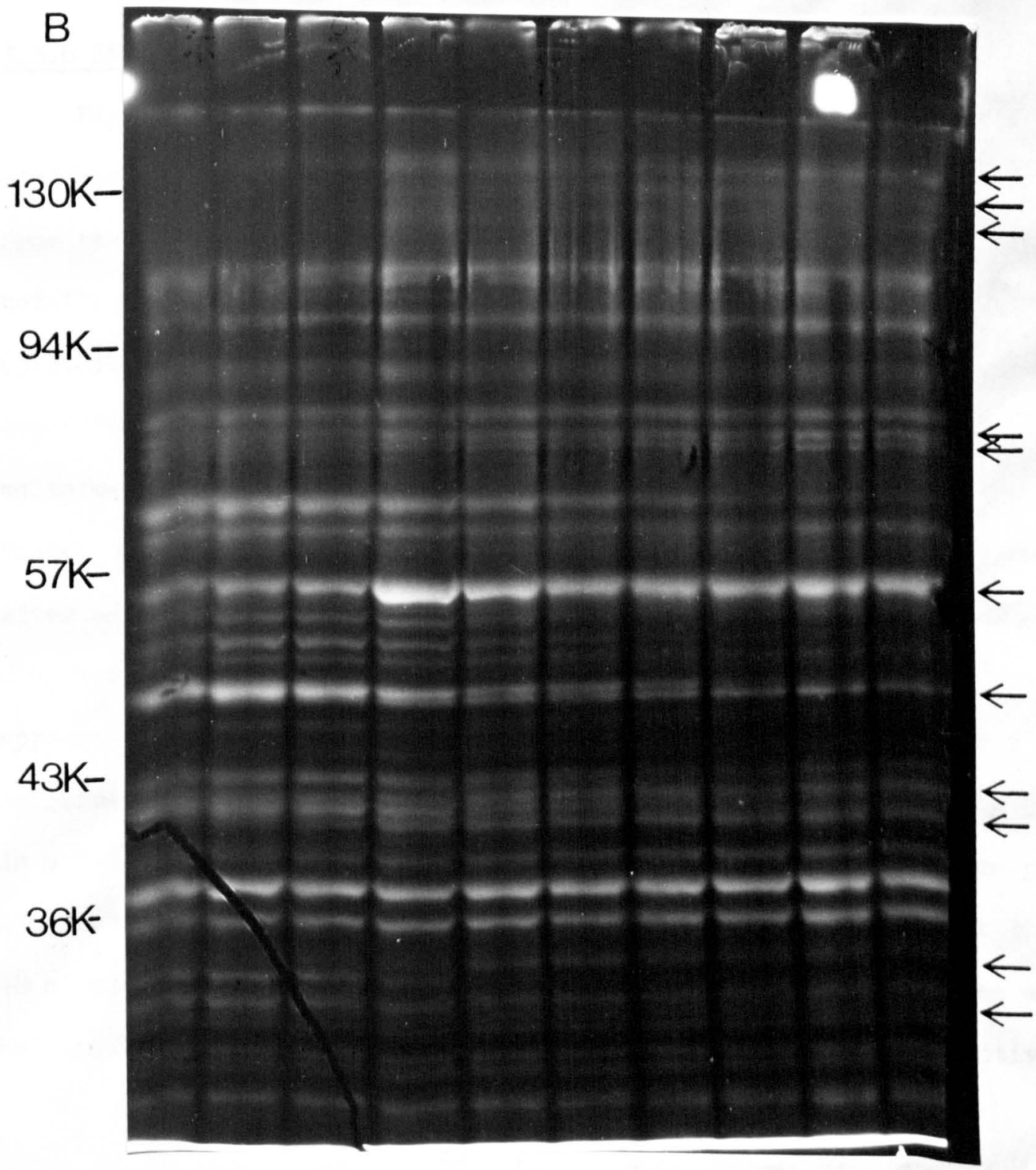
Figure 19: Linear sucrose gradient separation of cdc7.4 nuclei and analysis of proteins by SDS-polyacrylamide gel electrophoresis.

A 100ml culture of cdc7.4 was grown overnight to a final cell density of 1.8×10^7 cells/ml in YESD-AU_{10} medium containing 2 μCi/ml 6-^{3}H uracil and 0.1 μCi/ml(U-^{14}C) protein hydrolysate (2.1). A 2ml lysate was prepared as described in the legend to Figure 17, and loaded onto an 11ml linear 1.5M-2.4M sucrose gradient in PMN buffer, which was centrifuged in a Beckman SW40 rotor at 20K rpm for 15 minutes at 4°C. 1ml samples were removed from the gradients by displacement from below and precipitated overnight at -32°C by the addition of 2.3ml chilled absolute alcohol. The precipitates were taken up in 0.1ml Studier sample buffer (2.10)and boiled for 2 minutes. Figure 19a shows the analysis of these samples for radioactivity present in DNA  , protein  (2.6). Figure 19b shows the same samples electrophoresed on a 10% SDS-polyacrylamide gel (2.10). Bands which were observed to change as the gradient was descended, identifying proteins which were nucleus specific are marked with an arrow (←——).

A


Total ^{14}C cpm in protein x 10^{-5}
Total ^{3}H cpm in DNA x 10^{-3}
1 2 3 4 5
2 4 6 8 10
1 2 3 4 5 6 7 8 9 10
Fraction number

B


130K
94K
57K
43K
36K
bands enhanced in nuclear fractions

nucleus specific.

Such patterns were confirmed by SDS-polyacrylamide gel electrophoresis of S and p_2 fractions. Samples were obtained from cultures grown at 23°C, 38°C and in some cases 38°C shifted down to 23°C in the presence of (U-^{14}C) protein hydrolysate. Unfortunately, difficulty was found in reproducibly obtaining good p_2 fractions (as determined by DNA and protein recovery) from cultures grown at 38°C, again caused by variable susceptibility to lysis during spheroplast formation

An analysis of S and p_2 fractions using one-dimensional SDS-polyacrylamide gel electrophoresis will be presented later (4.3.2.6). This system represents a good analysis of some nuclear proteins in S.cerevisiae.

3.3.6 Effect of thiolutin on RNA and DNA synthesis in cdc7.4.

Figure 20 compares the effects of thiolutin (5µg/ml) and cycloheximide (100µg/ml) on DNA synthesis in cdc7.4 cultures released from the temperature block in the presence of inhibitor. Thiolutin totally blocked the cycloheximide-resistant burst of DNA synthesis (3.3.2). However, this level of thiolutin was found to cause immediate cessation of both RNA and DNA synthesis in exponentially growing cultures between 5 x 10^6 and 10^7 cells/ml (Figures 21 and 22). Protein synthesis continues at a decreasing rate for up to 40 minutes after addition of thiolutin at 2.5µg/ml or 5µg/ml (data not shown).

What is the significance of these observations? Figure 21 appears to show a direct effect of thiolutin on DNA synthesis. The antibiotic was shown to have no effect on DNA polymerase activity (2.14) in a 0-50% ammonium sulphate fraction of a crude extract from log-phase A364A cells (Figure 23). Preincubation of the enzyme fraction at 30°C for 10 minutes with 25µg/ml thiolutin had no effect on DNA polymerase activity, but this level was reported to totally inhibit RNA polymerase activity

Figure 20: Effect of thioluti n on the burst of DNA in synchronised cdc7.4 cells.

The experimental protocol was identical to that of Figure 12, with the following exceptions: an 80ml culture was synchronised using 0.8ml α-factor in methanol ($\triangle$) and after removal of α-factor the entire culture was shifted to 38°C ($\bullet$); after 105 minutes at 38°C the culture was split into 4x15ml portions, one remained at 38°C ($\bullet$), the other three were shifted to 23°C, one without inhibitor ($\circ$), one containing 100 µg/ml cycloheximide ($\square$) and one containing 5 µg/ml thiolutin ($\blacktriangle$). Thiolutin was madeup as a 2.5mg/ml solution in DMSO. The control cultures ($\circ$) and ($\bullet$) each received 30 µl DMSO at the time of addition of inhibitors.

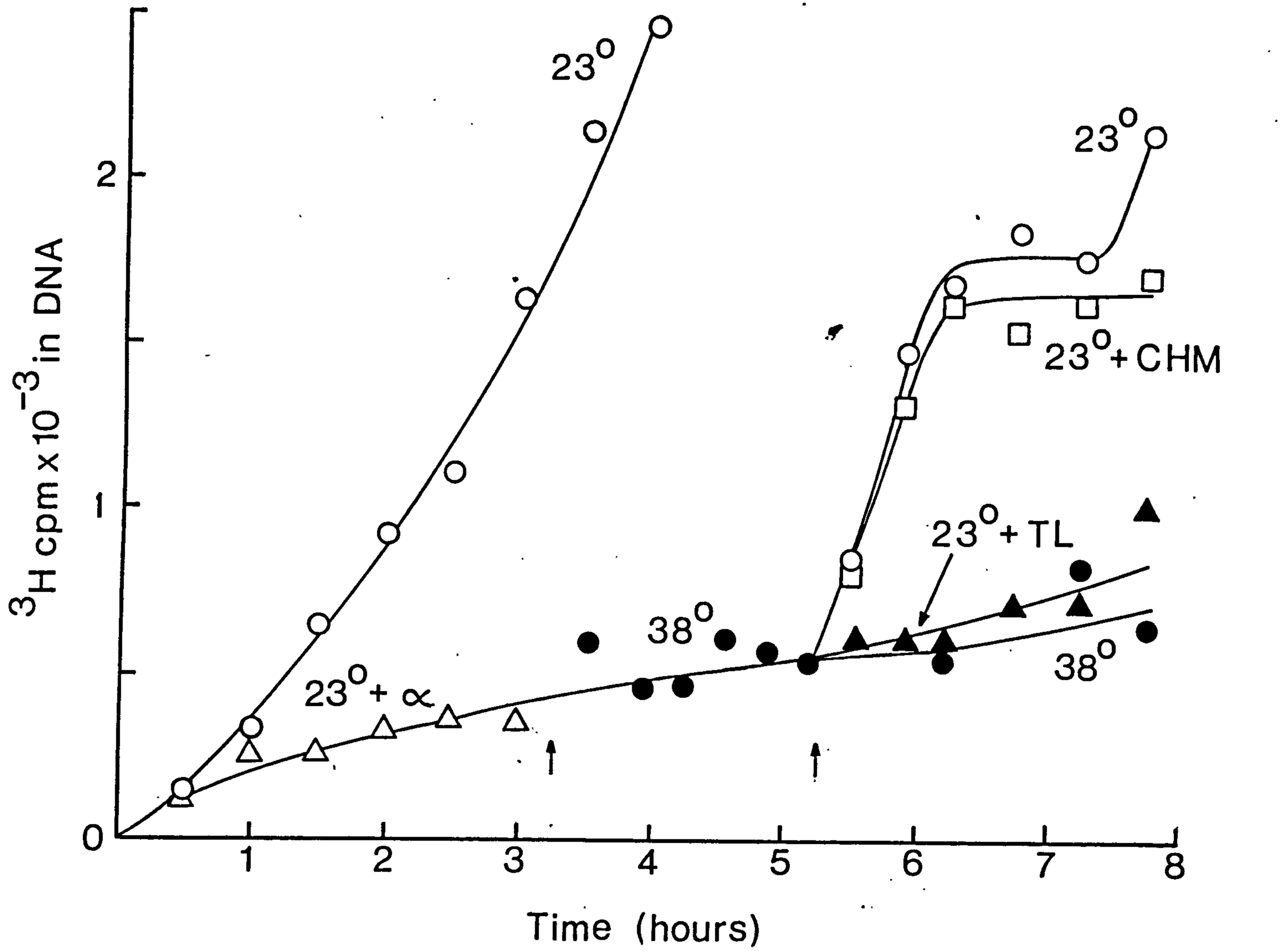

^{3}H cpm x 10^{-3} in DNA
0
1
2
1
2
3
4
5
6
7
8
Time (hours)
23°
23° + ∝
38°
23° + CHM
23° + TL
38°

Figure 21: Effect of thiolutin on DNA synthesis *in vivo*.

A 25ml log-phase culture of cdc7.4 in YESD-AU_5 (prepared by growth overnight in YESD-AU_{10} followed by centrifugation in an MSE bench centrifuge and resuspension of the cells in YESD-AU_5 at a density of $8x10^6$ cells/ml then grown at 23°C for 1 hour to allow exponential growth to resume) received 2.5 μCi/ml 6-^{3}H uracil, and 0.5ml samples were removed at the times indicated for radioactive incorporation into DNA (2.6.1). After 1 hour, the culture was split into two 10ml portions, one of which received 20 μl of a 2.5mg/ml solution of thiolutin in DMSO (● final 5 μg/ml), and the other received 20 μl DMSO (○). Cell viability was not affected by thiolutin at up to 4½ hours after addition in this experiment.

Figure 22: Effect of thiolutin on RNA synthesis *in vivo*.

0.1ml samples from the same cultures used in Figure 21 were processed for radioactive incorporation into RNA (2.6.2).

Figure 23: Effect of thiolutin on DNA polymerase activity *in vitro*.

A crude extract was prepared from log-phase cdc7.4 cells (2.12) and a 0-50% ammonium sulphate fraction was obtained. The ammonium sulphate precipitate was taken up in TEGD buffer (2.12) and dialysed. The fraction was diluted to a protein concentration of 5mg/ml with TEGD buffer and 45 μl aliquots were pretreated at 30°C for 10 minutes with either no additions (△); or 5 μl of a 0.25mg/ml solution of thiolutin in DMSO (□); or 5 μl of DMSO (○). After 10 minutes, 5 μl of the 0.25mg/ml thiolutin solution was added to the sample which had been incubated without additions (△), and 200 μl DNA polymerase assay mix (2.14) was added to each sample. Incubations were continued at 30°C and at the indicated times 50 μl aliquots were removed and processed as described in 2.14.

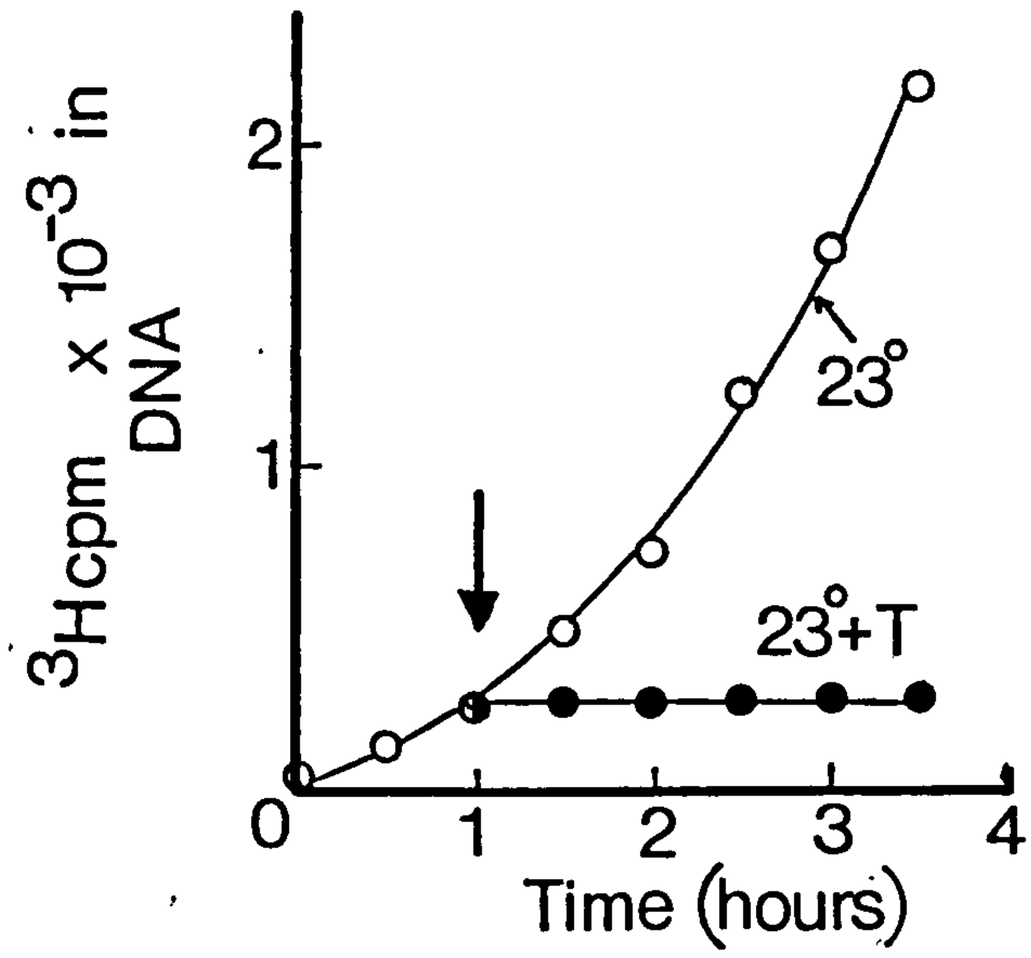


Fig.21

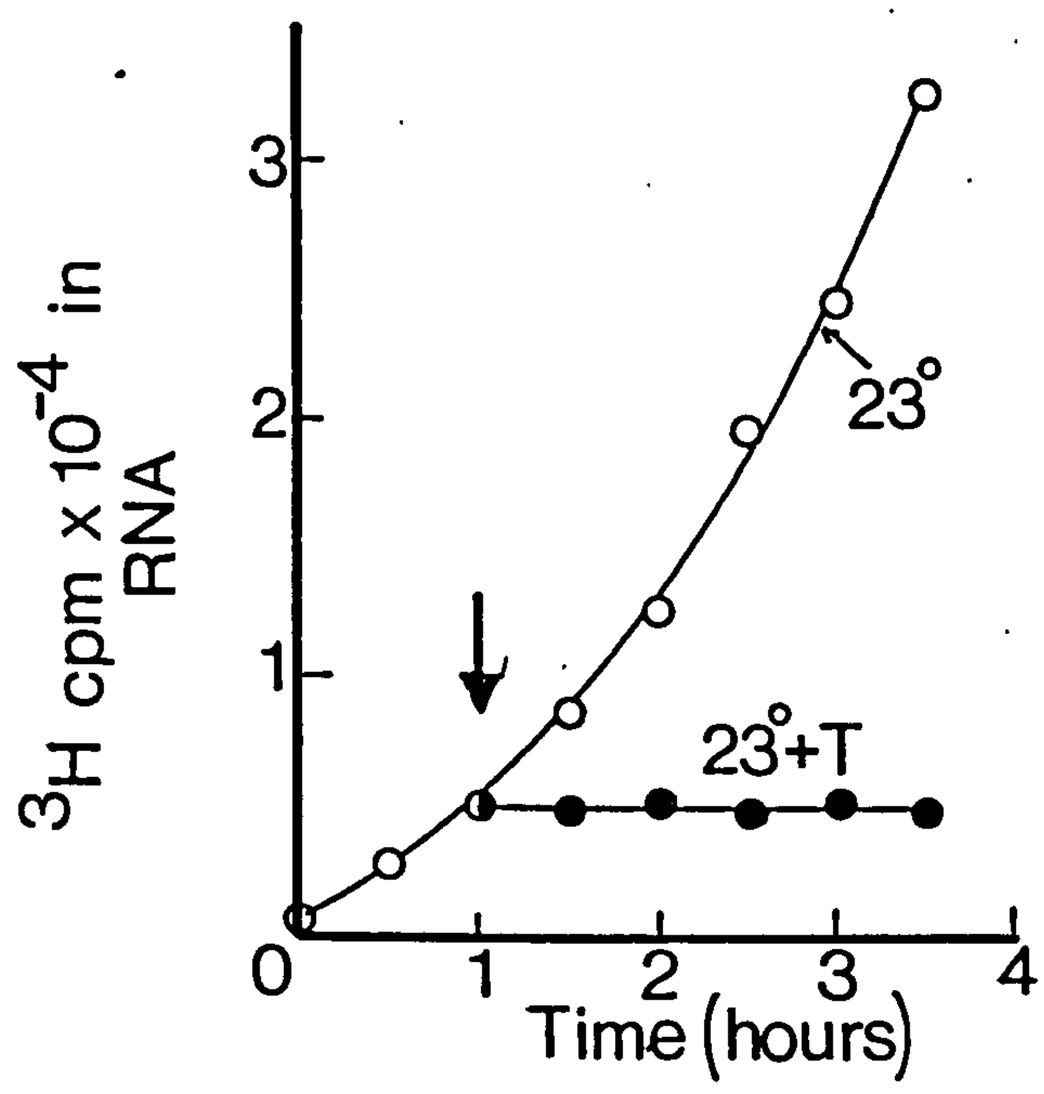


Fig.22

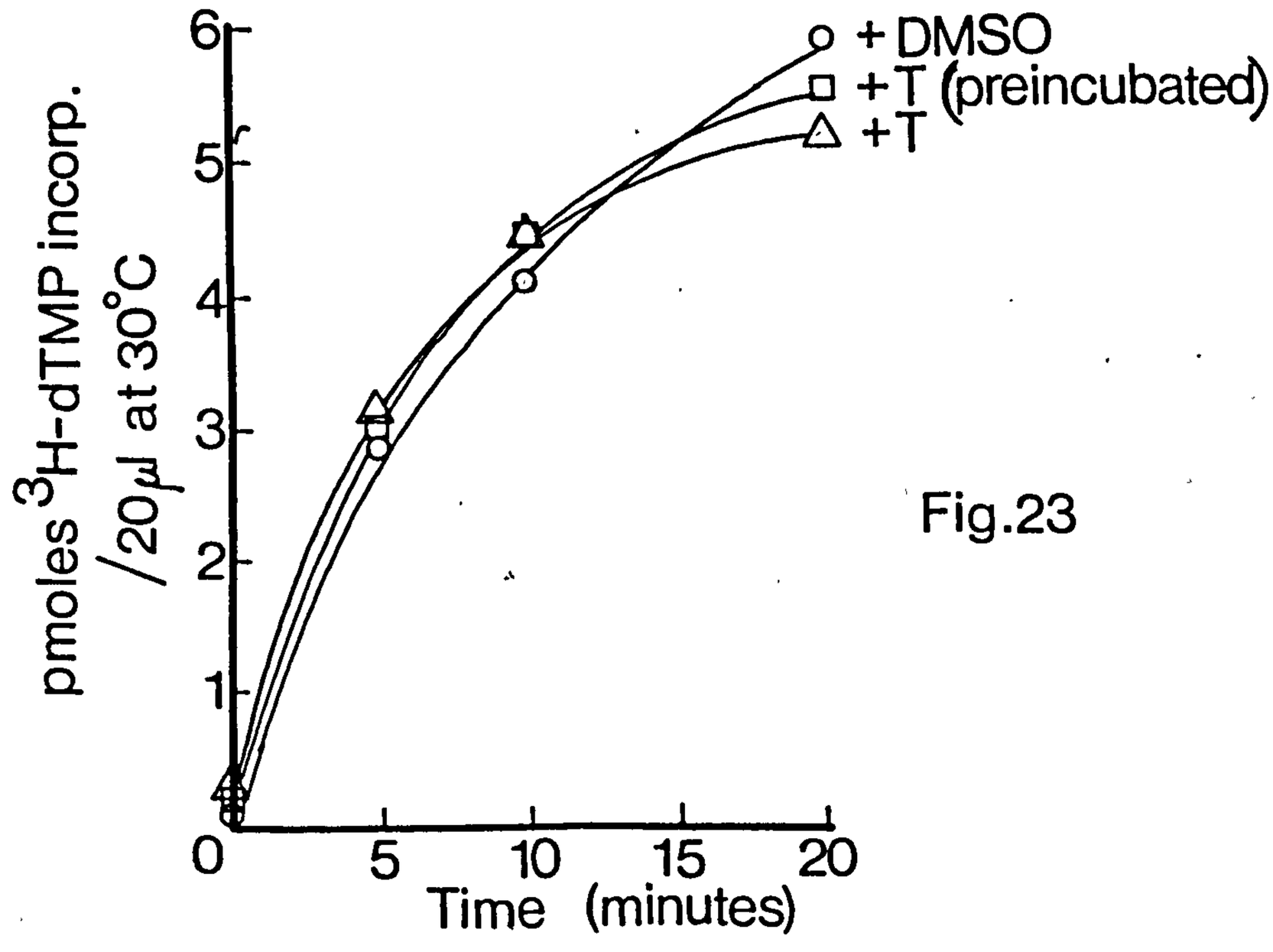


Fig.23

(Tipper, 1973).

The possibility, therefore, arises that thiolutin may affect an RNA priming step essential for ongoing DNA replication. Blumenthal and Clark (1977) showed nascent fragments in Drosophila cells to be short, in 3 major size classes of 61, 125 and 240 nucleotides (i.e. 4-6S). This contrasts with nascent DNA in prokaryotes, where fragments are 8-10S i.e. 1,000-2,000 nucleotides (Anderson, 1978). Yeast appears to be a typical eukaryote in this respect, generating short 4S fragments in vitro, (Oertel and Goulian, 1977) and also in vivo in ligase deficient strains, (Johnston and Nasmyth, 1978). Assuming thiolutin prevents their initiation, the residual DNA synthesis of nascent Okazaki fragments would not be detected in incorporation experiments such as Figure 21. (1ml of culture at $5x10^6$ cells/ml would incorporate at most 0.2 pmole of nucleotide if synthesis is discontinuous on both strands). It is, of course, possible that thiolutin in vivo may inhibit a component of the replication apparatus other than DNA polymerase. Therefore these results cannot unequivocally confirm a role for RNA synthesis in DNA replication in S.cerevisiae.

It must be concluded that thiolutin is not, as yet, as useful a probe of yeast DNA replication as rifampicin has proved in bacteria (Lark, 1972; Zyskind et al, 1977). It would be of great importance to distinguish "Grand Start" priming events from the priming of Okazaki fragments, as has been done in prokaryotes. A suggestion that actinomycin D can achieve this in CHO cells (Guy and Taylor, 1978) must be treated with caution, since the antibiotic interacts with DNA not with any RNA synthesising enzyme (Wells and Larson, 1970). However, strains of S.cerevisiae sensitive to low concentrations of actinomycin D do exist (Schindler and Davies, 1975), and it would be interesting to discover the behaviour of the cdc7.4 mutation in such backgrounds in the presence of inhibitor.

3.4 Conclusion.

The characterisation work reported in this Chapter has extended knowledge of the cdc7.4 mutation. Several points can be made. 1: The use of 6-^{3}H uracil to label DNA in yeast was shown to be a valid technique. 2: The DNA labelled after release from the α-factor block was shown to be nuclear. 3: DNA synthesised at the restrictive temperature in cdc7.4, and at the α-factor block was found to be predominantly mitochondrial. Shortly after this work had been performed, Newlon & Fangman (1975) reported the same result using the cdc7.4 temperature block. Thus mitochondrial DNA replication is independent of the CDC7 gene product (and also the CDC28 and CDC4 gene products) and α-factor mediated cell arrest. In later work on *in vitro* DNA synthesising systems (Chapter 6), this fact necessitated the use of a ρ° strain to eliminate incorporation. 4: The cdc7.4 lesion did not affect gross RNA and protein synthesis over a time scale in which the lesion in DNA synthesis was apparent. 5: There are no gross differences in phosphoprotein labelling in this time scale, suggesting that major protein kinase activities function normally. This does not exclude the possibility of a highly specific protein kinase being defective. 6: The decrease in phospholipid synthesis in cdc7.4 (H201.14.4) following recovery from α-factor arrest was not reproduced in cdc7.4 (DE200.1.3), suggesting that this is not an effect of the lesion in DNA synthesis. 7: A technique was developed which yielded an enriched nuclear fraction. 8: Thioluti.n, an inhibitor of RNA synthesis in yeast (Jimenez *et al*, 1973) and RNA polymerase *in vitro* (Tipper, 1973), was shown to totally inhibit DNA synthesis in cdc7.4 *in vivo* at low levels, without any effect on DNA polymerase *in vitro*. This is circumstantial evidence of an involvement of RNA synthesis in DNA replication, which must await definitive characterisation of the antibiotic for its final proof.

CHAPTER FOUR.

A study of cdc7.4 using polyacrylamide gel electrophoresis.

CHAPTER FOUR

A STUDY OF cdc7.4 USING POLYACRYLAMIDE GEL ELECTROPHORESIS

4.1. INTRODUCTION

The fact that cdc7.4 does not require protein synthesis to complete a round of DNA synthesis upon a shift from the restrictive to the permissive temperature (3.3.2.1.) suggests that the mutation is due to a temperature-dependent conformational change in protein structure. SDS-polyacrylamide gel electrophoresis was used to search for a mutationally altered protein. Various *in vivo* labelling protocols were used because such an altered protein might show abnormal rates of synthesis, processing or turnover. (Goldberg and Dice, 1974), or it might show net charge or size heterogeneity. Temperature-sensitive RNA polymerase mutants have been found in *E.coli* (Kirschbaum *et al* 1975), which show altered synthesis and degradation of defective β and β' subunits at the nonpermissive temperature. In this case, the defective protein was poorly represented in the cell, but unusually high levels might also be anticipated, as is the case in temperature-sensitive mutants of SV40 gene A, coding for T-antigen, a protein known to promote viral DNA replication, in which the overproduction of a 100,000MW protein was observed under restrictive conditions (Tegtmeyer *et al* 1975).

cdc7.4 was known to be inviable after only relatively short periods at high temperature (3.3.1.1.), so changes in protein profiles compared to the wild type parent A364A were expected. At present, there are at least 7 different alleles of the cdc7 gene in existence, (Hartwell *et al* 1973), some of which have not yet been studied. Five of these alleles were available, and their protein profiles were compared on one dimensional SDS-polyacrylamide gels, and also on two dimensional polyacrylamide gels.

Gross incorporation of ^{32}P-phosphate into phosphoprotein was found

not to be temperature-sensitive in cdc7.4 (3.3.4.1). However, there are good reasons for implicating a protein kinase in an initiation scheme. In certain cases a cell cycle dependence of protein kinase activity has been established. Hardie et al (1976) discovered two histone kinase activities in Physarum polycephalum nuclei which both act on histone H1 in G2 phase but show different times of appearance. Costa et al (1976) studied Type I and Type II cAMP dependent protein kinase activities during the cell cycle of Chinese Hamster ovary cells, and observed little Type II activity during G1 but a rapid increase at the G1/S boundary. More recently further evidence for the importance of protein phosphorylation in DNA synthesis initiation was given by the demonstration of protein kinase and ATPase activities in a protein antigenically related to SV40 T-antigen (Tjian & Robbins 1979). The phosphoproteins of A364A and cdc7.4 were analysed on SDS-polyacrylamide gels, using the labelling regime described earlier (Fig 15; 3.3.4.1.).

An anomalous protein of 72K MW was observed in cdc7.4 (H201.14.4) which was tested for correspondence with lesion in DNA synthesis by genetic analysis.

4.2 MATERIALS AND METHODS.

4.2.1 Gel Electrophoresis.

One dimensional SDS gels were run as described in 2.10.

Two dimensional gels were run by the methods of O'Farrell (1975) and Ames and Nikaido (1976).

(i) Sample preparation. About 10^8 cells were harvested and washed with double-distilled water. Pellets were resuspended in 0.05 ml sonication buffer (10mM Tris-H61 pH 7.4, 5mM $MgCl_2$; 50µg/ml RNAase A, 2mM PMSF.) and transferred to 50mg No.100 glass beads (acid washed) in Eppendorf tubes, then sonicated at 0°C for 3x15 second intervals with the microprobe on an MSE sonicator, chilling for 10 seconds between each burst. 3µl of 1mg/ml EP-DNAseI was added, and the sample digested for 50 minutes in ice. Finally, 75µl Buffer A (9.5M urea, 2%w/v Triton X-100 (Sigma), 2% Ampholines composed of 1.6% pH range 5-7, 0.4% pH range 3-10, 5%β-mercaptoethanol, stored frozen in aliquots)was added, and the sample heated at 75°C for 10 minutes. Samples were assayed for TCA-precipitable counts at this stage, and equivalent counts were loaded onto each gel.

(ii) First-dimension gels. The first, isoelectric focussing dimension, was run in disc gels of 2.5 mm diameter. Gels were composed of the following mix (sufficient for eight gels) :- 5.5 g urea (Ultra-Pure), 1.33 ml acrylamide stock (28.38% acrylamide, 1.62% bis-acrylamide), 2ml 10% TritonX-100, 1.97 ml double-distilled water, 0.415 ml Activator solution (0.14 mg/ml riboflavin, 1% TEMED) 0.4ml pH 5-7 ampholines, 0.1 ml pH 3-10 ampholines (both LKB-Produkter). The gel mix was filtered through Whatman GF-C, degassed and run into acid washed siliconised precision-bore glass tubes, the bottoms sealed with parafilm. Gels were overlaid with 8M urea and polymerised by exposure to fluorescent light for 1½ hours. Gel length was 11.4 cms.

The urea overlay was removed prior to sample application, and following this, a fresh overlay replaced. The cathode solution was

0.02N NaOH, and the anode solution 0.01N Phosphoric acid. Gels were run for at least 5000 Volt hours (350 volts for 18 hours).

For transfer to the second dimension, gels were removed and equilibrated in SDS sample buffer (62.5 mM Tris pH 6-8, 10% v/v glycerol, 0.7 M-mercaptoethanol, 2.3% SDS, 1 mM PMSF) and frozen at -20°C until required.

(iii) Second dimension. 10% SDS-polyacrylamide gels of 1mm thickness were made as in General Methods. The stacking gel was 5% polyacrylamide filled to within 2mm of the top of the glass plates. This gap was filled with 1.5% agarose (Sigma) in SDS sample buffer and bromophenol blue, the disc gel laid on, and completely covered with agarose. Gels were run for 5 hours at 18mA, or overnight at 50 volts.

After running gels were fixed in 500 ml 10% Acetic acid, 10% isopropanol.

(iv) Fluorography. This was performed on all 2-D gels to enhance sensitivity of autoradiography. The method is that of Bonner and Laskey (1974) with modifications by J. B. Taylor (personal communication). (a) After draining off the HAc-isopropanol, the gels were given two washes in 150 ml DMSO previously used in (b), 20 minutes per wash. (b) A further 20 minute wash in 250 ml fresh DMSO. (c) Gels were soaked for 4 hours at room temperature in 60ml, 10% PPO in DMSO, warming occasionally to keep the PPO in solution. (d) PPO was precipitated in the gel by washing with water, removing the excess PPO by hand with a rubber glove. (e) Gels were dried down as described, as soon afterwards as possible.

4.2.3 Genetic Analysis.

The techniques and theory of yeast genetics were used as described (Mortimer and Hawthorne, 1969). Construction of diploids from haploid strains of opposite mating types by growth on selective media, was followed by sporulation and microdissection of the resulting tetrads.

Media used were described in the Cold Spring Harbor Manual of Yeast Genetics (1975).

(i) Construction of diploids.

Diploids resulting from a cross between two strains were selected by mixing streaks of fresh growths of each from YEPD-AU agar plates onto MV-agar containing any mutally required supplements. Colonies were subcultured on the same plates prior to use.

(ii) Sporulation of diploids.

A single diploid colony was picked off and serial diluted to about 100 cells/plate on presporulation agar (0.8% yeast extract, 0.3% peptone, 10% dextrose). Colonies were grown for 1-2 days on this rich medium at 30°C, then clumps of cells were transferred by sterile toothpick to sporulation agar (1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, plus mutual supplements). After at least 5 days at 30°C on this medium, the presence of asci can be seen under the microscope.

(iii) Dissection of asci.

β-glucuronidase (snail gut juice) attacks the ascus wall, leaving the spore coat undamaged (Wright and Lederberg 1957). Dissection of digested asci was performed by Dr. D. Wilkie (Botany and Microbiology Dept., UCL). The resulting tetrads were grown on YEPD-TAU plates.

4.3 RESULTS AND DISCUSSION.

4.3.1. One dimensional PAGE analysis.

4.3.1.1. Amino acid labelling experiments.

The patterns of proteins synthesised at the permissive temperature 23°C, the restrictive temperature 38°C and upon a shift from 38°C to 23°C, were analysed on one dimensional SDS-polyacrylamide gels by autoradiography. Fig 24 shows the marked difference between stained and labelled bands and the effect of temperature shifts on cultures of cdc7.4. The broad, ill defined appearance of the bands was found to be due to the use of thick gels (1.6mm) causing band spreading upon drying. This was improved in later experiments by using thinner gels (0.8mm).

In early experiments, no differences between A364A and cdc7.4 extracts were observed.

Fig. 25 shows an autoradiograph of a later gel on which were run whole cell extracts of proteins labelled at 23°C and at 38°C, in cultures of A364A, cdc4.3, cdc7.4 (4008), cdc7.4 (H201.14.4), cdc28.1. A band appeared at 72K MW which was specific to cdc7.4 grown at 23°C. A fuller analysis of this band is presented in 4.3.2. Estimates of molecular weight were obtained from a calibration curve. With the exception of the band at 72K MW, no band was observed in cdc7.4 extracts which could not be reproduced in corresponding A364A extracts. Studies on the temperature dependence of protein synthesis in S.cerevisiae (Marmiroli et al 1976) have indicated that the optimal temperature for mitochondrial protein synthesis is 37°C and for cytoplasmic protein synthesis is 30°C with glucose as carbon source. Thus major metabolic differences are to be expected between 23°C and 38°C. These differences are reflected in the altered pattern of proteins synthesised at the two temperatures. (Examples are arrowed on Fig.25). The pattern changes presented a major problem in the search for an altered protein.

Figure 24: SDS-polyacrylamide gel electrophoresis of whole cell extracts from cdc7.4 cultures.

A 30ml log-phase culture of cdc7.4 in YESD-AU$_5$ was prepared as described in the legend to Figure 21. The culture was split into three 10ml portions, two of which were shifted to 38°C, and one remained at 23°C. One of the 38°C cultures and the 23°C culture received 1 µCi/ml (U-^{14}C) protein hydrolysate (2.1). Incubation was continued for 2 hours. After this time radioactive label was added to the previously unlabelled 38°C culture, and this was shifted to 23°C for 2 hours. The cells were removed by centrifugation at 4°C in an MSE bench centrifuge, washed once in Studier sample buffer (2.10) and then resuspended in 0.1ml of the same buffer containing 1mM PMSF. The samples were transferred to polycarbonate tubes containing 0.25g acid washed No.100 glass beads (BDH) and sonicated for 1 minute using the microprobe on an MSE sonicator whilst chilling in ice. A further 0.15ml sample buffer was added, and after centrifugation to remove debris the clear supernatant was treated for 2 minutes in a boiling water bath. After assaying for radioactivity present in protein, 25 µl samples (containing 9500cpm) were electrophoresed on a 10% SDS-polyacrylamide gel (2.10). After staining, the gel was autoradiographed as described in 2.19.

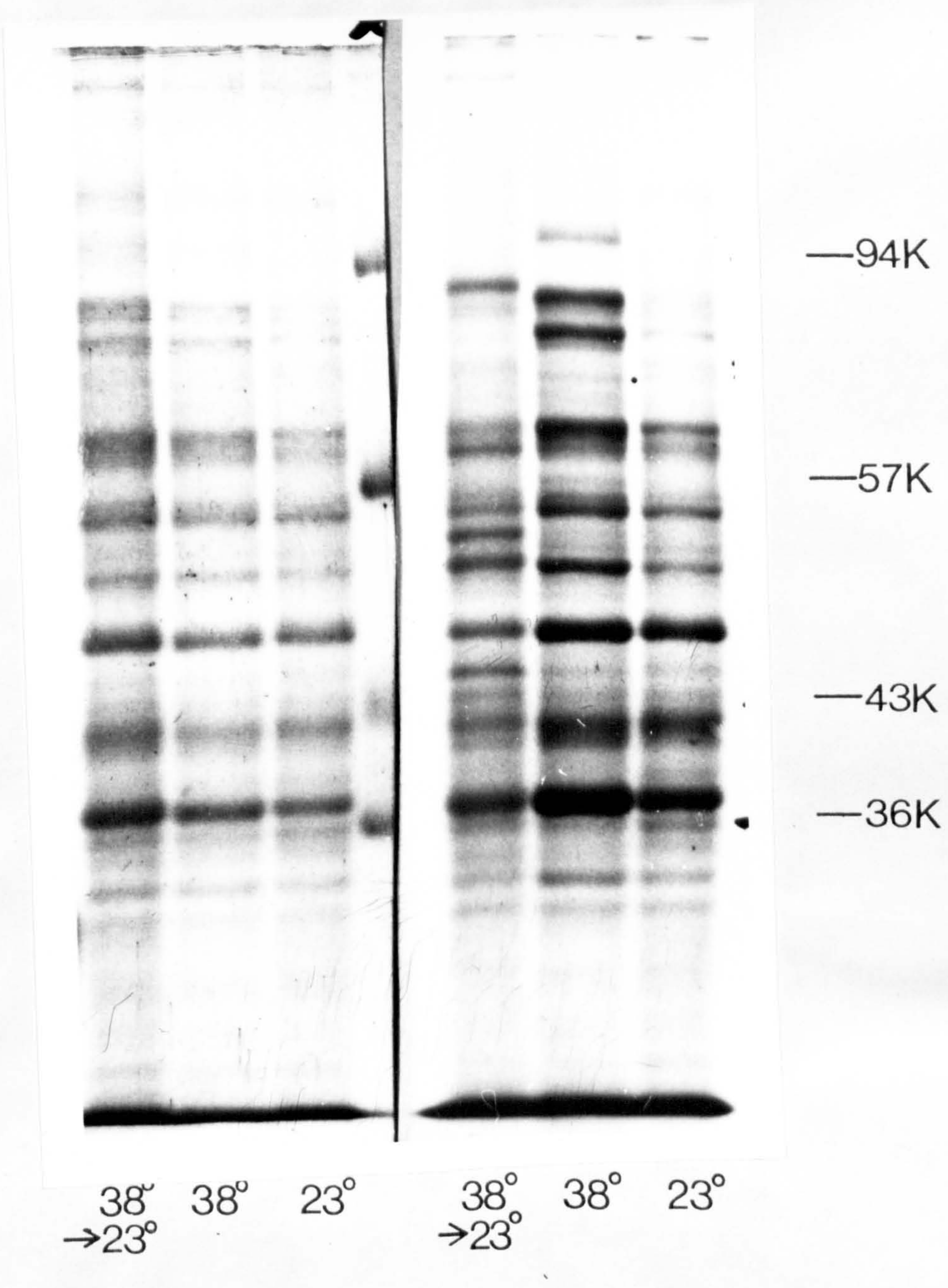
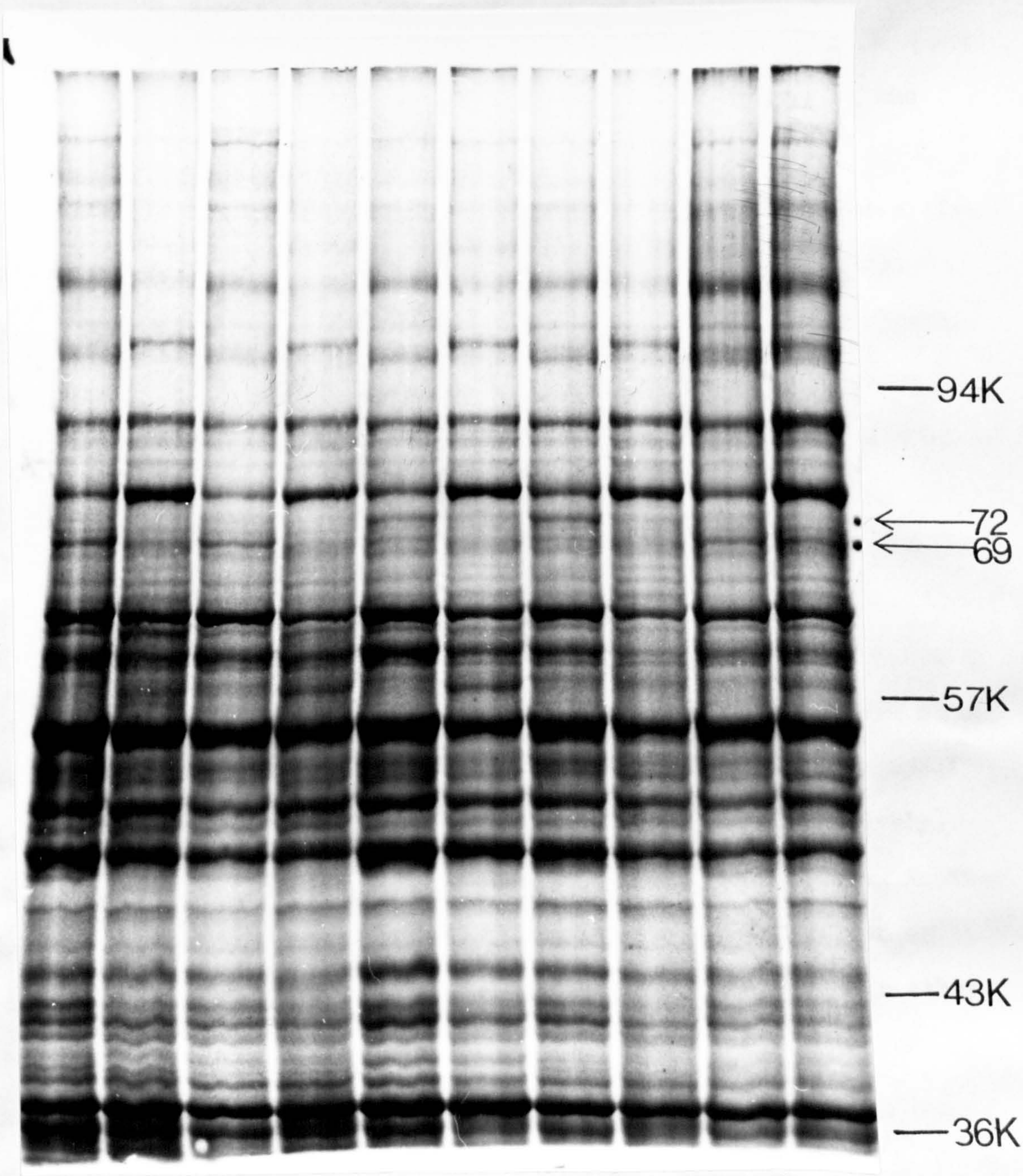

STAINED
AUTORAD.
—94K
—57K
—43K
—36K
38°→23°
38°
23°
38°→23°
38°
23°
TEMP °C

Figure 25: Comparison of protein profiles of whole cell extracts from A364A, cdc4.3, cdc7.4 (4008), cdc7.4 (H201.14.4) and cdc28.1 cultures by SDS-polyacrylamide gel electrophoresis.

The culture conditions, radioactive labelling and sample preparation were the same as in Figure 24 except that only cultures labelled at 23°C and 38°C were used, and the level of (U-^{14}C) protein hydrolysate was 0.38 Ci/ml.

The standards used were: phosphorylase a (P_a, 940000MW); pyruvate kinase (PK, 57000MW); ovalbumin (430000MW); lactate dehydrogenase (LDH, 36000MW). In later experiments, β-galactosidase (β-gal, 130000MW) was also used. A calibration curve was prepared for each gel

Two anomalous bands in cdc7.4 cultures at 72K and 69KMW are indicated by arrows.


94K
72
69
57K
43K
36K
23° | 38° | 23° | 38° | 23° | 38° | 23° | 38° | 23° | 38°
A364A
cdc4.1
cdc7.4
4008
cdc7.4
H201.14.4
cdc28.1

4.3.1.2. ^{32}P labelling of proteins.

Proteins phosphorylated during and after release from α-factor treatment were studied. The experiment involved pulsing cultures grown in low Pi medium with ^{32}P phosphate for 15 minute intervals at 38°C and 23°C, 1 hour and 2 hours after release from the α- block, using the protocol of Fig 15. It was known from the kinetics of uracil incorporation into DNA (3.3.2.1.) that at approximately 45 minutes after release the cells synchronously commence a burst of DNA synthesis. Thus the cdc7.4 gene product is present and functional at 23°C at this time. After 2 hours the cells have finished the burst of synthesis, whilst cdc7.4 cells kept at 38°C are beginning to lose viability (3.3.1.1). These conditions were chosen to optimise the differences not only between A364A and cdc7.4, but also between the restrictive and permissive temperatures in cdc7.4.

The results of one such experiment, not reproduced here because of excessive background on the autoradiogram, drew attention to five bands. Band I at 72K was the stained band present in cdc7.4 but not in A364A (4.3.1.3.). Two bands (bands II and V, at 51K and 34K respectively) were more prominently labelled at 38°C in cdc7.4 than in A364A. Another two bands, III and IV at 49K and 45K respectively, were not seen as labelled bands after 2 hours at 38°C in cdc7.4, but were seen in other treatments. Any of these anomalies could have been due to the lesion. However, on careful repetition of this experiment several times, these features were not reproducible. One experiment is shown in Fig 26.

Band V appeared strongly labelled in all treatments, and migrates in the same region as histones, (Thomas & Färber 1976). Band II did not become more prominently labelled. Bands III and IV showed variable labelling, but not in a strain or temperature dependent fashion. Unlike the protein phosphorylation patterns seen in less complex systems, for example T7 (Rahnsdorf et al 1974; Zillig et al 1975), the results in this simple enkaryote are nowhere near as clear cut. However, the band

Figure 26: Phosphorylated proteins of A364A and cdc7.4.

Cultures of A364A and cdc7.4 were pulsed for 15 minutes with ^{32}P phosphate at the times shown in Figure 15 to yield samples 1, 2, 3, 4 and 5. Additional samples were also obtained in this experiment (numbers 6, 7, 8 and 9). These corresponded to: 6, 3 hours at 23°C after release from α-factor; 7, 3 hours at 38°C; 8, 1 hour at 23°C after 2 hours at 38°C; 9, 1 hour at 23°C in the presence of 100μg/ml cycloheximide after 2 hours at 38°C. These culture conditions were equivalent to those used in the DNA synthesis experiments (Figures 12 and 20).

Whole cell extracts were prepared as described in Figure 24. Samples containing 34000 ^{32}P cpm in protein were run on the 2 SDS polyacrylamide gels shown (Note; sample 6(A) was overloaded).

Figure 26A shows the stained gels, and Figure 26B shows the resulting autoradiographs. A band at 46K (marked by an arrow in Figure 26B) appeared to be a protein phosphorylated specifically in the presence of α-factor.

A, A364A; 7, cdc7.4(H201.14.4).

Fig.26A

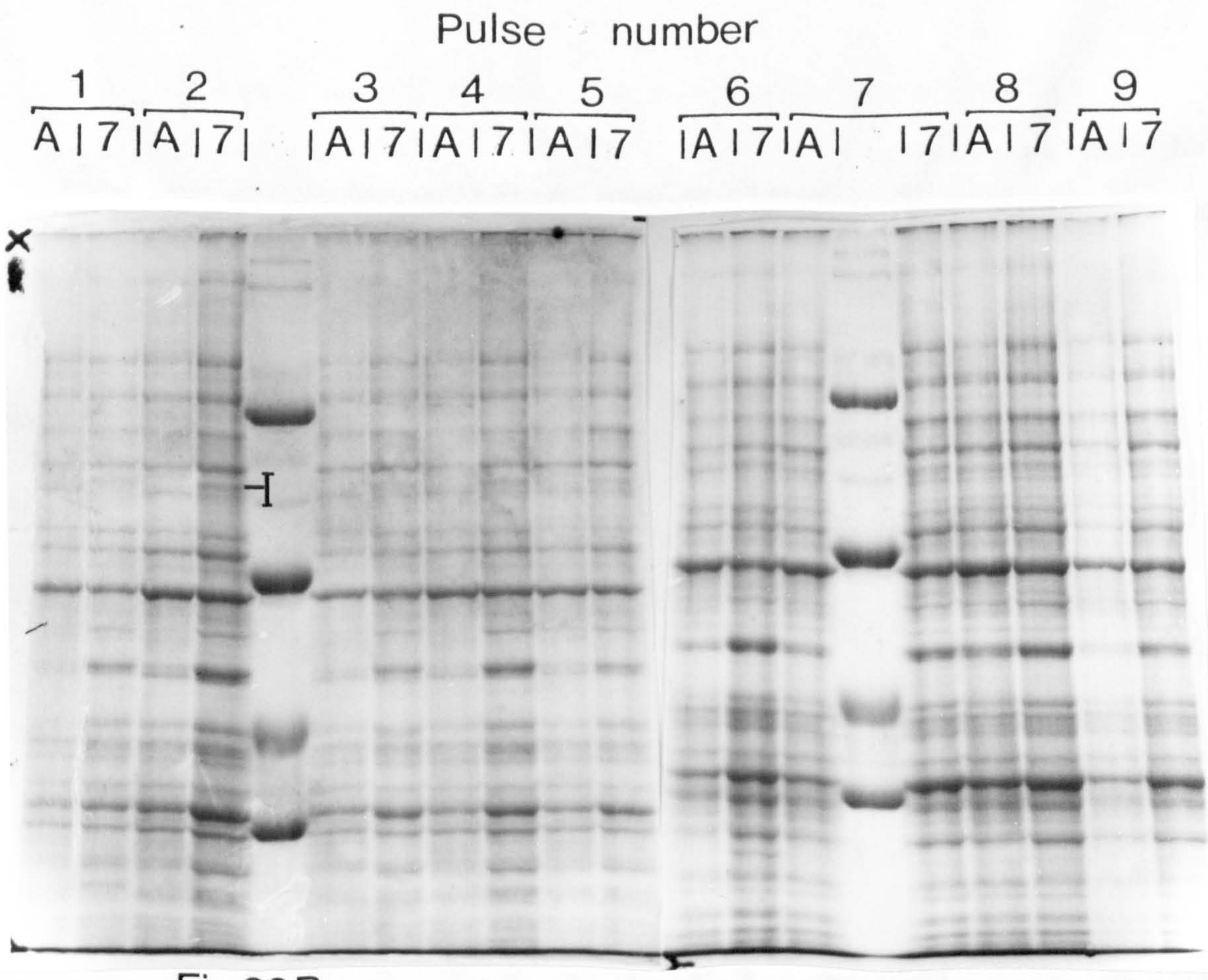
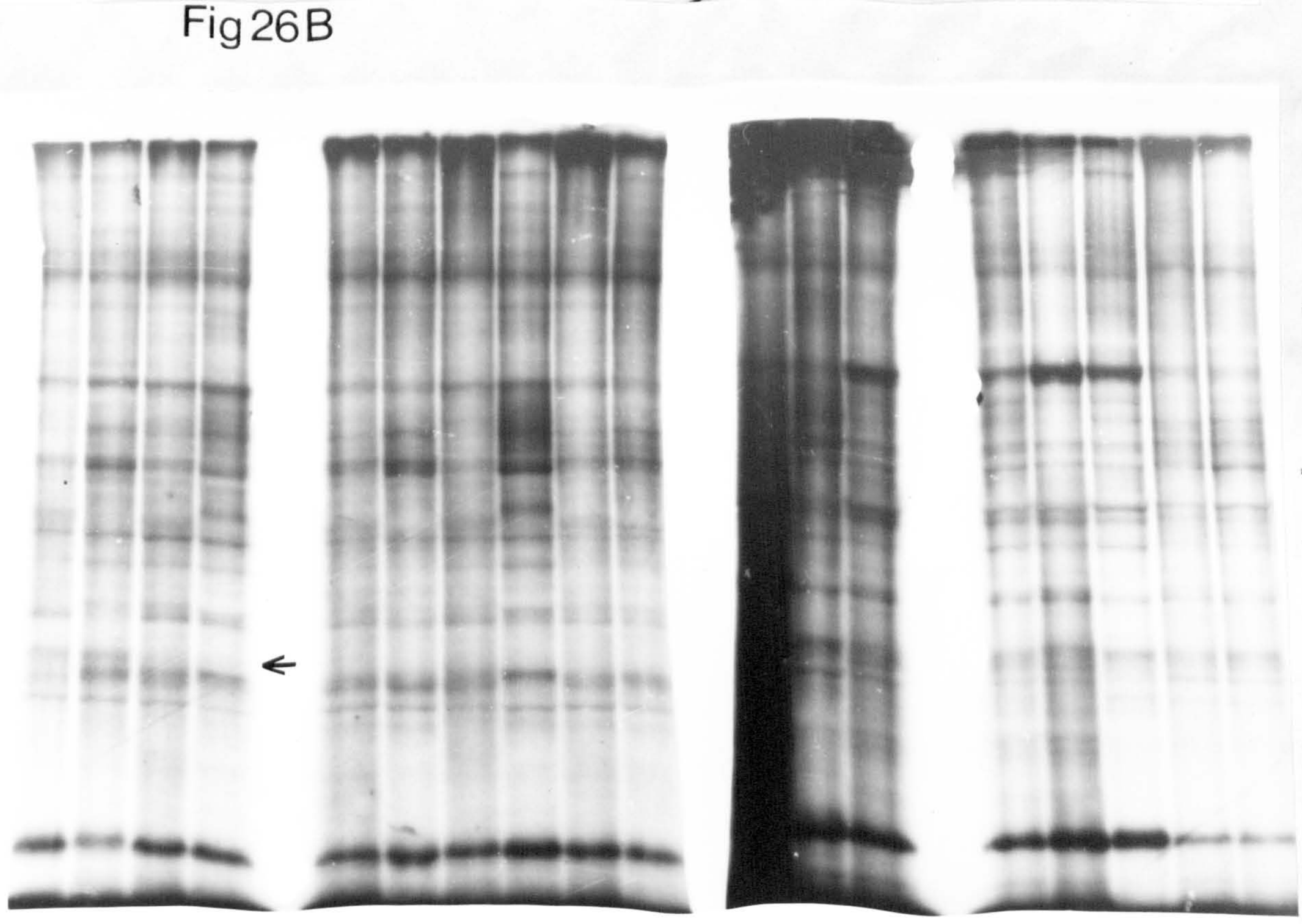


Fig 26B



at 72K (which does not appear to be a phosphoprotein) was a reproducible feature of cdc7.4 at 23°C. It is worth noting that the major stained band present on all SDS-polyacrylamide gels, tentatively identified as tubulin (Snyder and McIntosh,1976), which runs with an apparent molecular weight of 55K, was seen as a doublet of phosphorylated proteins on autoradiograms.

All gels of ^{32}P-phosphate labelled proteins had high backgrounds, which may be due to indiscriminate binding of nucleotides or free phosphate to protein. Dialysis of samples prior to electrophoresis did not improve the results. It was thought not to be caused by nucleic acid, since ultrasonicated DNA does not enter 10% polyacrylamide gels (Loening 1967).

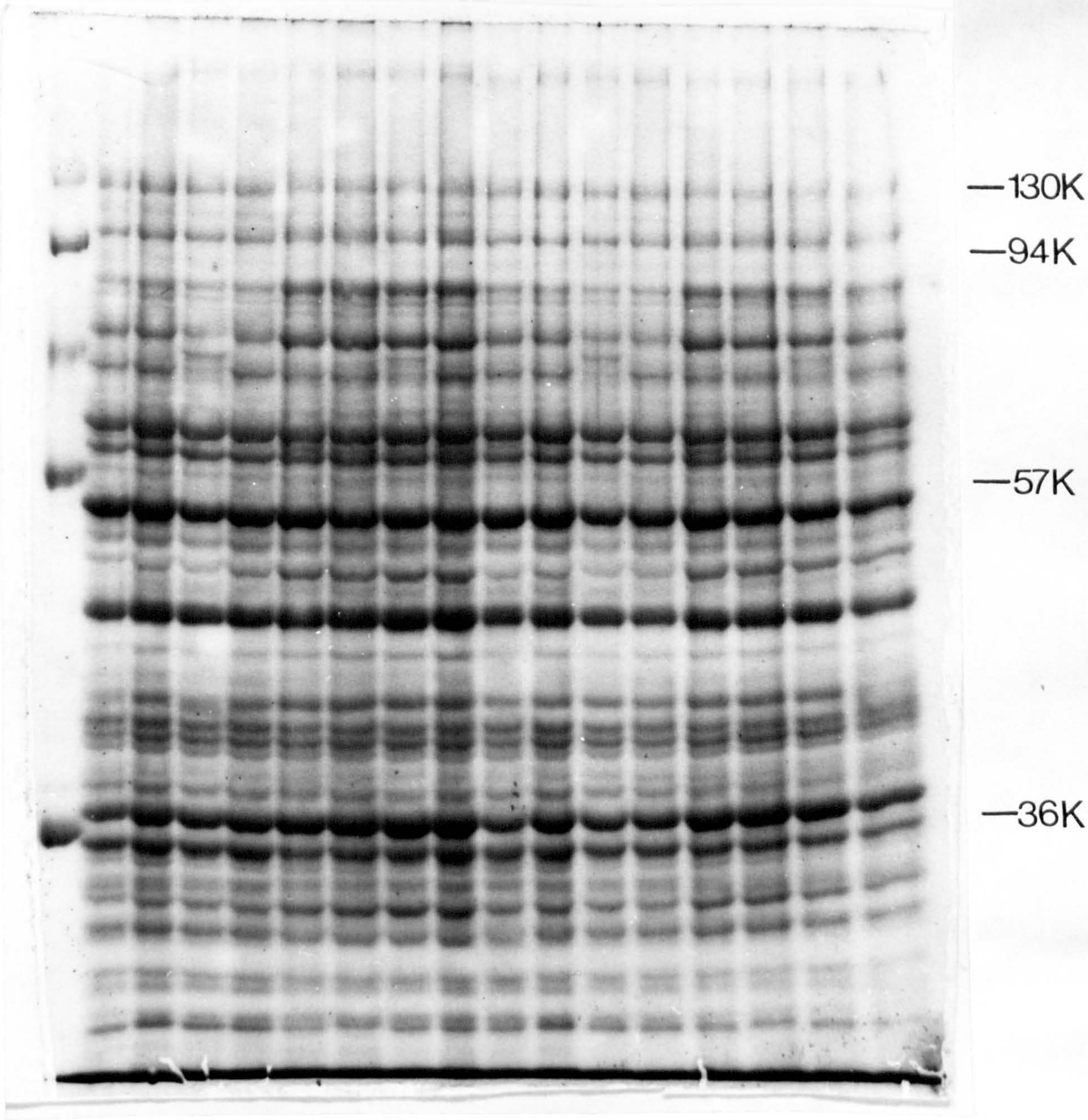
4.3.1.3. Effects of growth medium and temperature upon the 72K band.

At the time when these experiments were performed, the 72K band had only been seen during the ^{32}P-phosphate labelling experiments. It was therefore possible that the band arose in cdc7.4 due to growth in low Pi medium. SDS-polyacrylamide gels were run displaying unlabelled whole cell extracts of A364A, cdc4.3, cdc7.4 and cdc28.1 grown at 23°C and 38°C, using four sorts of growth medium, YEPD-AU, low Pi, YESD-AU$_{10}$ and SD-AU$_{10}$. Fig 27 shows the results for extracts from YEPD-AU and low Pi grown cells. There was no effect of growth medium upon the appearance of the 72K band (data for YESD-AU$_{10}$ and SD-AU$_{10}$ not shown). The band at 72K which was present in all cdc7.4 extracts at 23°C seemed to be reciprocally related to a prominent band at 69K in A364A, cdc4.3, and cdc28.1 which was only poorly represented in cdc7.4 at 23°C. Moreover, at 38°C, as shown in tracks 6-9 and 14-17, the apparent proportions of the two bands changed in cdc7.4. More of the 69K band appeared, with a reduction in the 72K protein. This suggested that the 72K protein was a unique feature of cdc7.4 and that it also responded to the temperature of growth.

Figure 27: Effect of growth medium and temperature upon the 72K MW band.

10ml log-phase cultures of A364A, cdc7.4, cdc4.3 and cdc28.1 were grown for 2 hours at 38°C and 23°C in YEPD-AU medium and low Pi medium (see Figure 15). Whole cell extracts were prepared and electrophoresed as described in Figure 24.

A, A364A; 4, cdc4.1; 7, cdc7.4; 28, cdc28.1.


—130K
—94K
—57K
—36K
A 4 7 28 A 4 7 28 A 4 7 28 A 4 7 28
23°C 38°C 23°C 38°C
YEPD–AU
low Pi

4.3.2. Genetic analysis of the 72K band present in cdc7.4.

The best method for testing whether an observed defect corresponds to a defined genetic lesion is to demonstrate cosegregation upon outcrossing (Mortimer and Hawthorne 1969). Consequently a prototrophic diploid colony (DE100) resulting from a cross between cdc7.4 (a ade ura his lys tyr) and S2072D (α arg leu thr trp) was selected by growth on minimal medium. After induction of sporulation, 4 tetrads were isolated (Fig 28). The resulting 16 strains were tested for temperature sensitivity of growth, effect of temperature on DNA synthesis, segregation of genetic markers, and the presence of the 72K band.

4.3.2.1. The effect of temperature on growth is shownin Fig 29. The figure shows the gradual loss of growth in specific strains as raised from 23°C to 30°C, 35°C, 37°C and finally 39°C. At 35°C and 37°C a 2:2 segregation of temperature sensitivity was observed. Strains which were sensitive to 37°C were designated at ts1. However, at 38-39°C, a further growth defect appears which results in eventual temperature sensitive segregation of 4:0. 3:1 (twice) and 2:2. S2072D and the diploid strain DE100 both grew satisfactorily at 38°-39°C. This indicates the presence of a second ts lesion. From Fig 29 it was possible to identify strains 100.1.2, 100.1.3, 100.2.1, 100.4.1 as carriers of this defect, which was designated ts2. Where cosegregation with the more drastic ts1 oocurred, as presumably in 100.3.1 and 100.3.3 and the parent cdc7.4, the presence of ts2 was completely obscured.

The four strains of tetrad 1 (4:0 segregation) were further tested by following the increase in cell number at 23°C and 38°C (Fig 30). Again two phenotypes were easily distinguishable. Strains 100.1.1 and 100.1.4 (both ts1) behaved in the manner of the cdc7.4 parent. Strains 100.1.2 and 100.1.3 (both ts2) reach cell numbers at 38°C not much lower than the wild type parent. These two strains unambiguously displayed the ts2 phenotype when grown on agar plates at 39°C (Fig 29).

Figure 28: Dissection of four tetrads from the cdc7.4a x S2072Dα cross.

After sporulation of diploid colonies, four tetrads were dissected. The 2:2 segregation of the adenine requirement is shown by the pink colouration of auxotrophic strains. The strains were numbered as follows using the first tetrad as an example: DE100.1.1; DE100.1.2; DE100.1.3; and DE100.1.4. (The number 100 following the DE prefix denotes that this was the first cross series using the original strains obtained from L.H. Hartwell, the second number is the tetrad number, and the third, the strain number.

Figure 29: Effect of temperature upon growth of the outcrossed strains (a).

Cells were plated out at approximately 10^6 cells/ml and grown on YEPD-TAU agar plates for 4 days at the temperatures specified below. The enlarged caption identifies the strains.

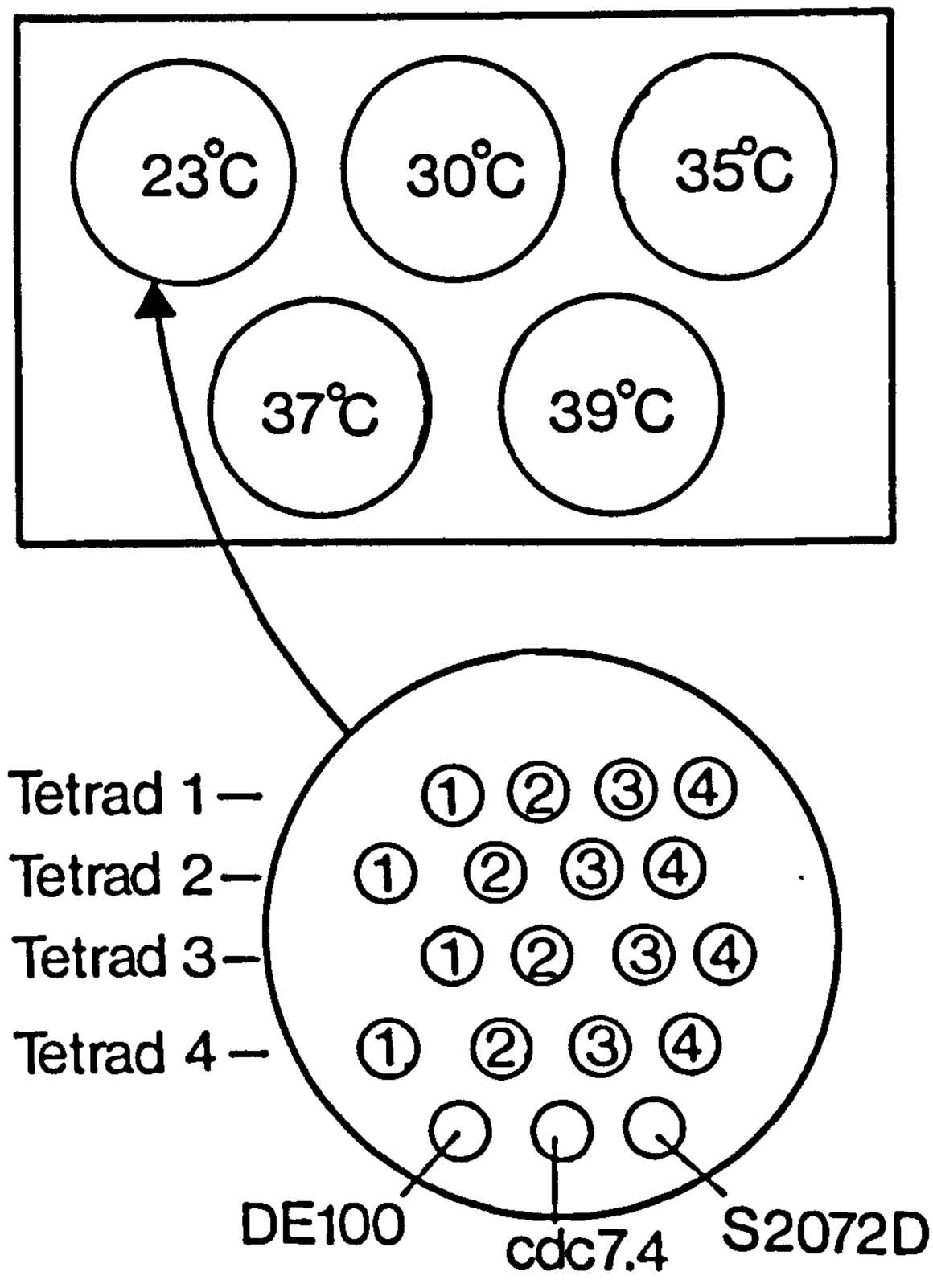


Figure 28

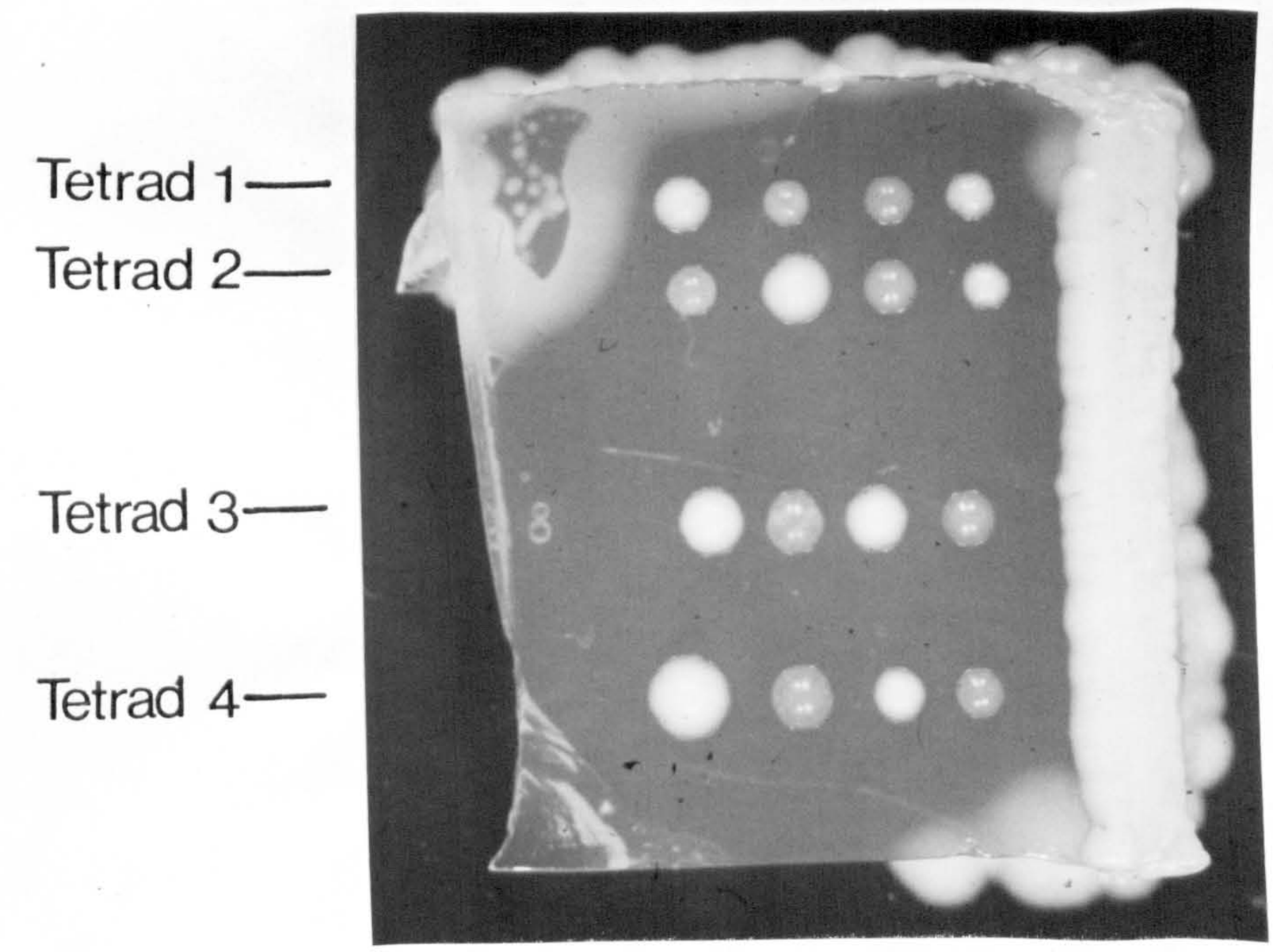


Figure 29

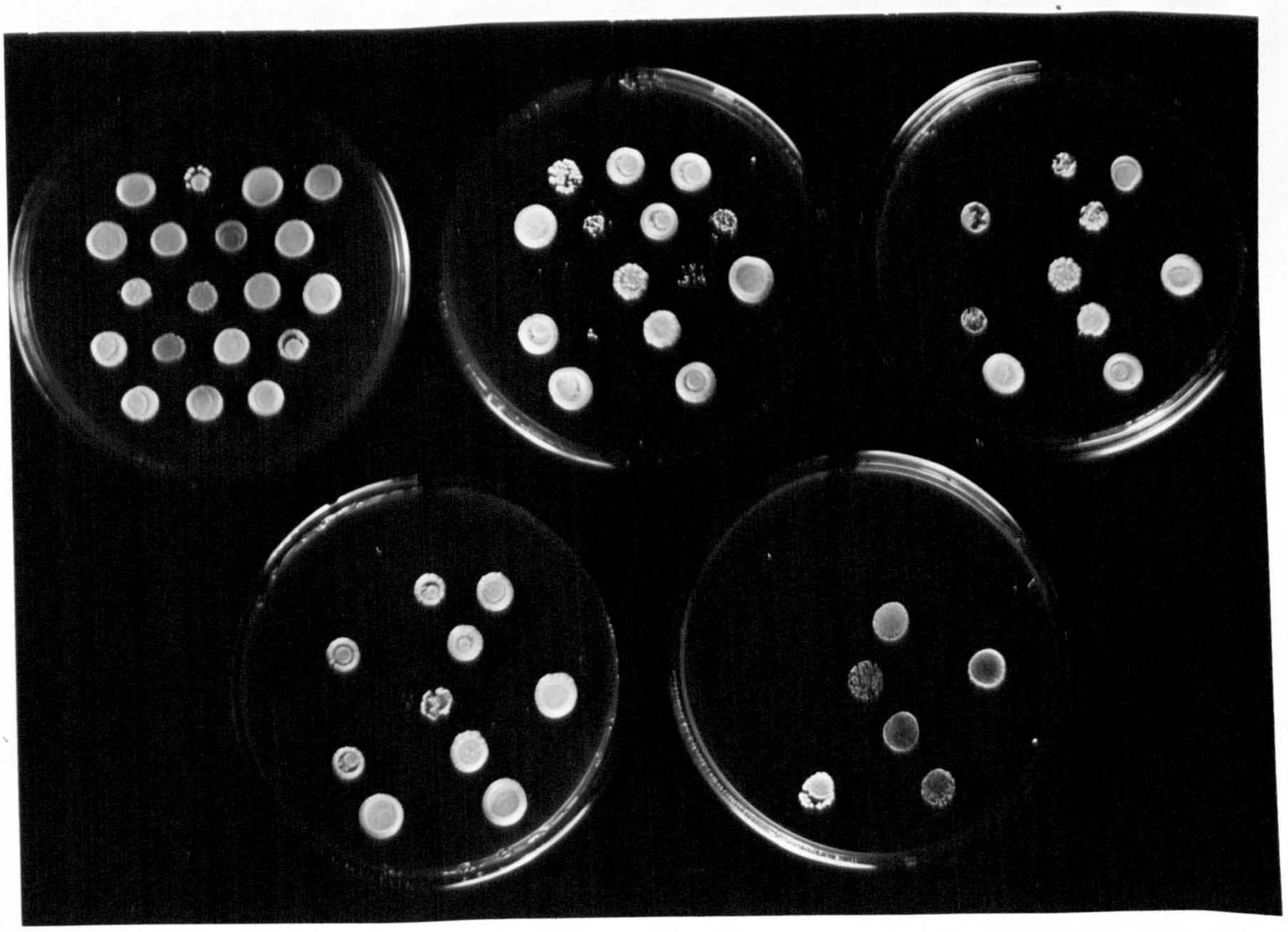
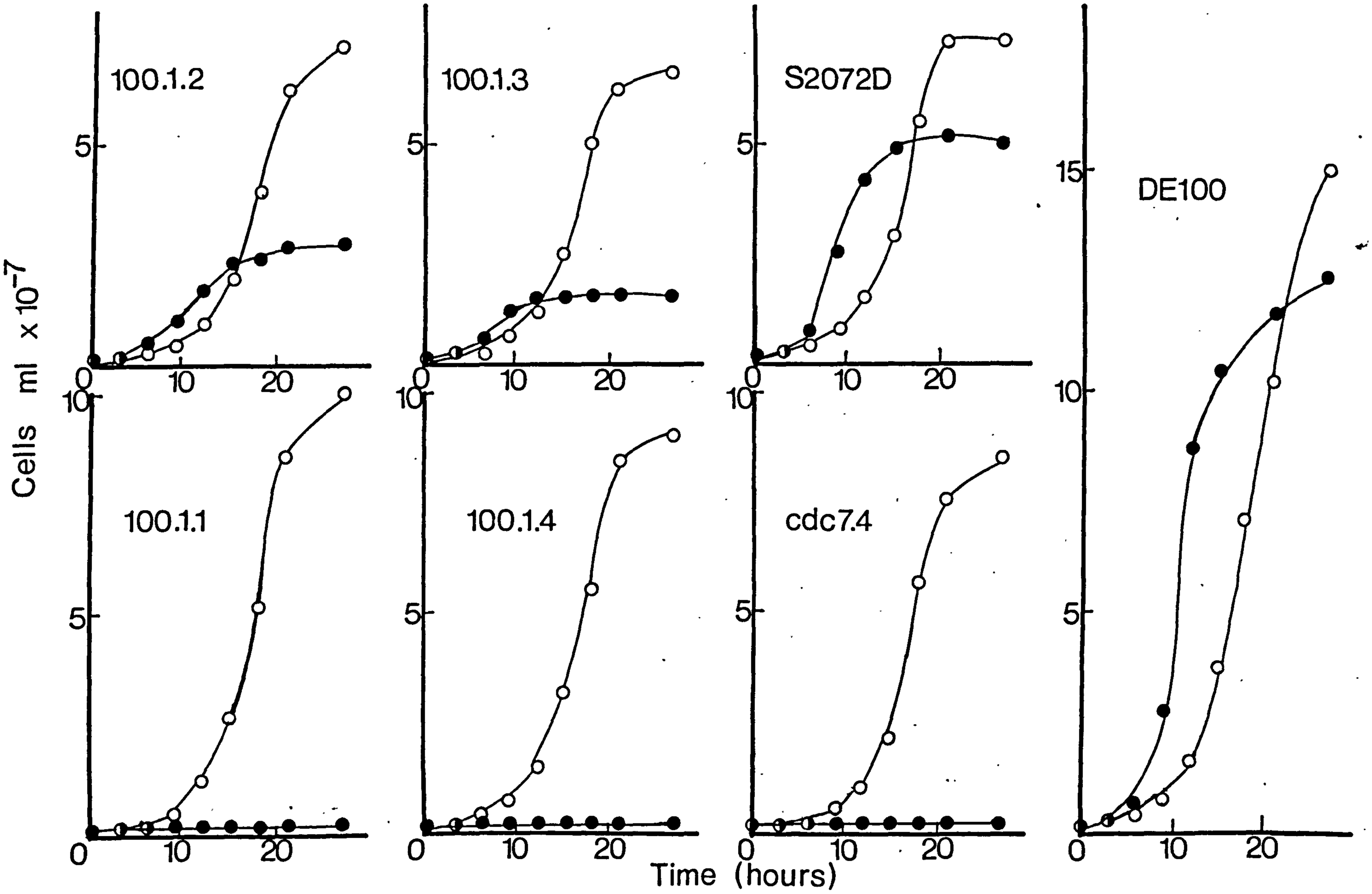


Figure 30: Effect of temperature upon growth of the outcrossed strains (b).

Cultures of strains 100.1.1, 100.1.2, 100.1.3, 100.1.4, cdc7.4, S2072D and DE100 (Diploid) were adjusted to 10^6 cells/ml in YESD-AU_{10} plus full amino acid supplements and grown at 23°C and 38°C. Cell number was measured by haemocytometer (2.5). [○] 23°; [●] 38°C


100.1.2
100.1.3
S2072D
DE100
100.1.1
100.1.4
cdc7.4
Cells ml x10⁻⁷
Time (hours)
0
5
10
15
20

This apparent discrepancy could be due to leakiness of the block at 38°C or a failure to give first cycle arrest, (Hartwell 1974). The latter is suggested by the fact that cells diluted to 10^4 cells/ml never reach 10^6 cell/ml even after prolonged incubation.

4.3.2.2. The effect of temperature on DNA synthesis was tested in a protocol derived from Hartwell (1967). Exponentially growing cultures of the 16 derived strains and the parent DE100, were split, one portion remaining at 23°C, and the other shifted to 38°C for 2½ hours. After this period, the 38°C culture was itself split, one half remaining at 38°C, the other shifted back to 23°C. These conditions were chosen to give optimal synchrony without excessive loss of viability (3.3.1.1.) such that the final temperature drop would give a burst of DNA synthesis in those strains harbouring a defect in the initiation of DNA synthesis (Hartwell 1967). Two distinct phenotypes emerged. The first was that which was expected for an initiation mutant. This behaviour correlated always with strains previously designated ts1. In the second case, DNA synthesis increased more rapidly at 38°C than at 23°C, decreasing on a shift down to 23°C, as typified by the diploid parent DE100. This was true of strains which were wild type for growth, as well as ts2 strains. 2:2 segregation of this character was upheld in all 4 tetrads. The results for tetrad 1 and the parent DE100 are shown in Fig 31. Cell viability estimations performed during this experiment showed 2:2 segregation of poor : good at 38°C. Two strains from each tetrad had essentially zero viability after 4½ hours at 38°C. This always corresponded with the presence of ts1.

4.3.2.3. The results of SDS gel electrophoresis of whole cell extracts of the strains is shown in Fig 32. The 72K band segregates 2:2 in all 4 tetrads (data for tetrad 2 not shown).

Figure 31: DNA synthesis in the outcrossed strains.

30ml exponential cultures of strains DE100.1.1, 100.1.2, 100.1.3, 100.1.4 and DE100 were prepared as described in the legend to Figure 21 received 5 Ci/ml 6-^{3}H uracil. The cultures were split into 20ml and 10ml portions, the larger cultures being shifted to 38°C, the smaller ones remaining at 23°C. 0.5ml samples were removed throughout the experiment at the times shown and processed for radioactive incorporation into DNA (2.6.1). After 2½ hours the 38°C cultures were split into two 8ml portions, one of which was returned to 23°C. [o], 23°C; [●], 38°C.

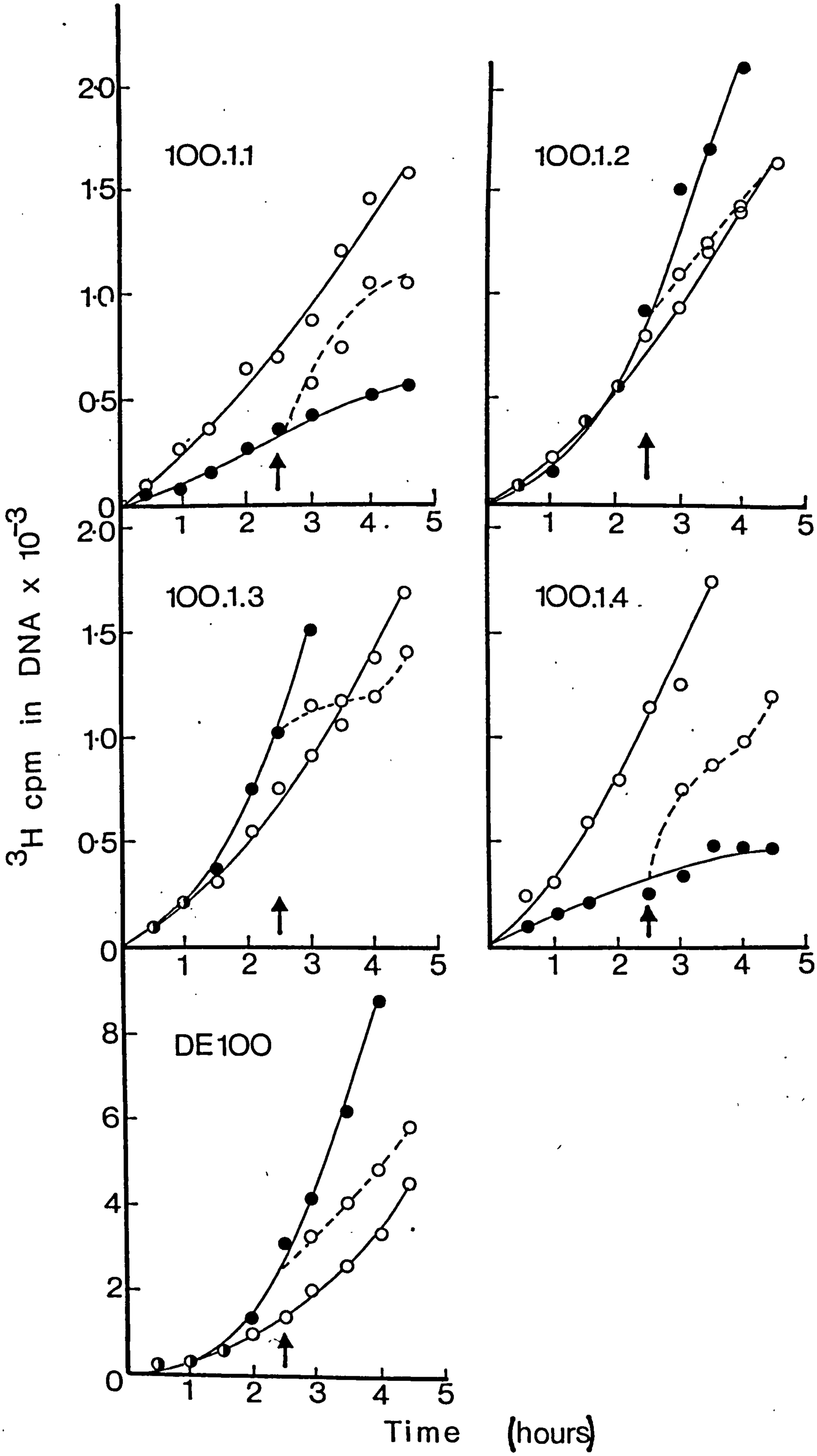
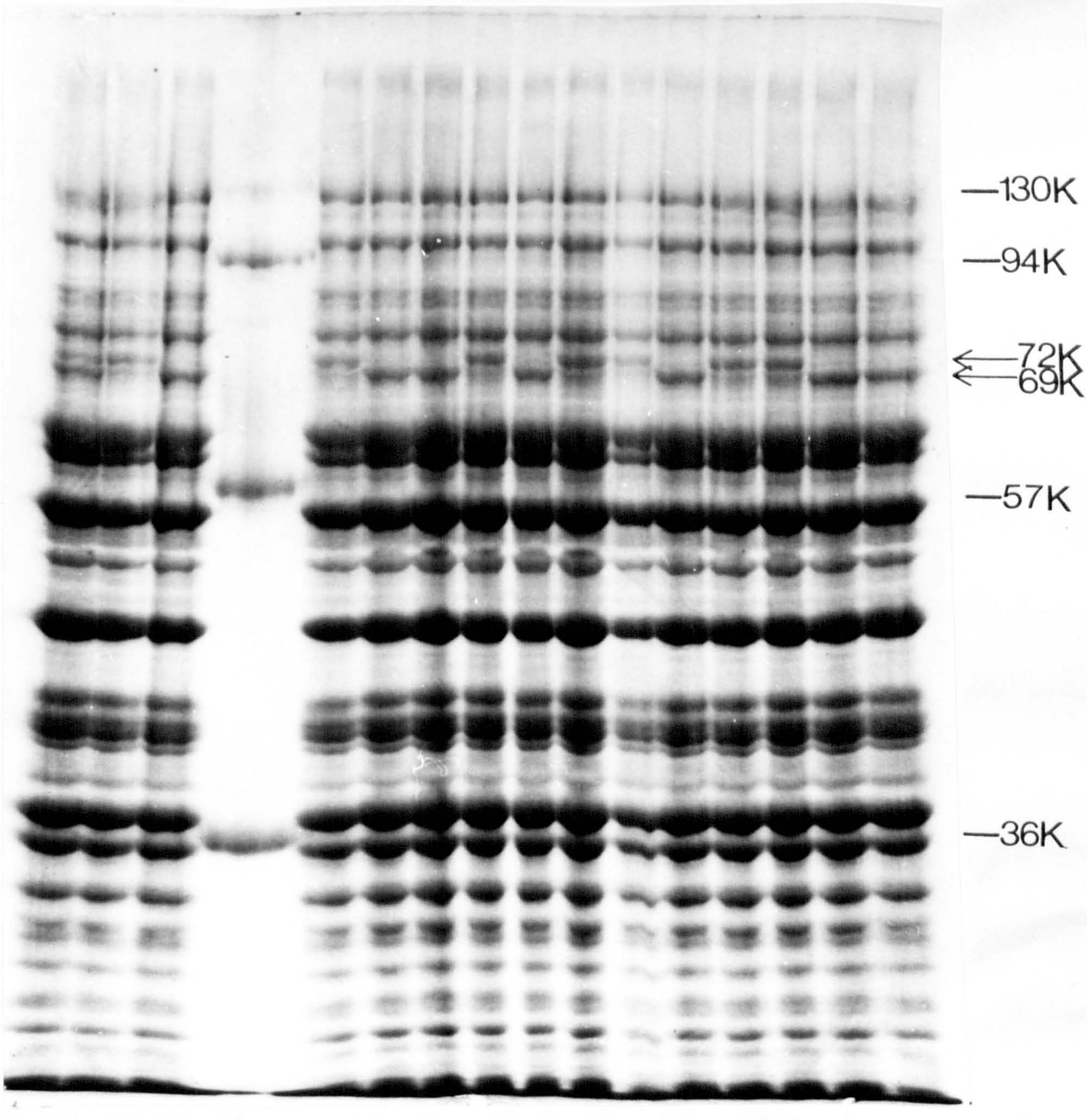

100.1.1
100.1.2
100.1.3
100.1.4
DE100
^{3}H cpm in DNA x 10^{-3}
Time (hours)
0
0·5
1·0
1·5
2·0
1
2
3
4
5
6
8

Figure 32: SDS-polyacrylamide gel electrophoresis of whole cell extracts of the outcrossed strains.

Whole cell extracts were prepared from 10ml cultures of the outcrossed strains in tetrads 1, 3 and 4, and also cdc7.4, S2072D and DE100, grown in YEPD-AU to $9x10^6$ cells/ml as described in the legend to Figure 24.



DE100 7 S | 1.1 | 1.2 | 1.3 | 1.4 | 3.1 | 3.2 | 3.3 | 3.4 | 4.1 | 4.2 | 4.3 | 4.4

Table VII: Summary of properties of the DE100 series outcrossed strains.

STRAIN	OBSERVED DEFECTS tsl	DNA initiation	ts2	72K MWband	GENETIC MARKERS
100.1.1	-	-	+	+	ura leu his, a
100.1.2	+	+	-	-	ura thr arg ade tyr his trp, α
100.1.3	+	+	-	-	lys ade thr arg trp, α
100.1.4	-	-	+	+	lys leu tyr, a
100.2.1	+	+	-	+	ura ade leu [tyr his trp]
100.2.2	-	-	(+or-)	+	lys thr arg
100.2.3	+	+	+	-	lys ade thr leu tyr his trp
100.2.4	-	-	(+or-)	-	ura arg [tyr his trp]
100.3.1	-	-	(-)	-	lys ura arg his
100.3.2	+	+	+	+	lys ade leu tyr trp
100.3.3	-	-	(-)	+	thr tyr his
100.3.4	+	+	+	-	ura ade thr leu arg trp
100.4.1	+	+	-	+	arg tyr trp
100.4.2	-	-	(+or-)	+	lys ura ade leu tyr
100.4.3	+	+	+	-	lys ura thr arg his
100.4.4	-	-	(+or-)	-	leu ade thr tyr his trp
cdc7.4 (H201.14.4)	-	-	(-)	+	ura ade his lys tyr, a
S2072D	+	nt	+	-	arg leu thr trp, α
DE100 (Diploid)	+	+	+	+and-	

Notes: + denotes wild-type phenotype, - denotes mutant phenotype

( ) denotes result which cannot be verified

[ ] denotes result uncertain due to inadequate growth.

4.3.2.4. Summary.

TableVII displays a complete analysis of genetic markers, presence of the 72K band, ts1 and ts2 in all of the strains used above. There was no correspondence of the 72K band with either ts2 or ts1. It was notable that the diploid parent of the strains appears to contain equal amounts of the 69K and 72K proteins.

TableVII shows that there was no strain which had the genotype $ts1^{(+)}$ $ts2^{(-)}$ $72K^{(-)}$. This strain, i.e. carrying only the lesion on the DNA synthesis was thought desirable for future study. To obtain it, a further cross between 100.1.1a and S2072Dα was constructed and the progeny (DE200) dissected as previously. The three tetrads isolated showed lower spore viability, two giving only 3 surviving colonies. Temperature sensitivity segregated 2:2 in the intact tetrad, expected for the presence of a single lesion. Two of these strains DE200.1.3a and DE200.3.2 behaved as ts1 when tested for growth at 38°C and also lacked the 72K band.

DE200.1.3 was tested in the same protocol as used earlier (3.3.2.1) to prove that the ts defect was still the same as the original cdc7.4. Fig 33 shows that this strain retains the insensitivity to cycloheximide, on dropping a synchronised culture from 38°C to 23°C. In all later studies on cdc7, DE200.1.3 was the strain used unless otherwise specified.

4.3.2.5. Confirmation of the uniquencess of the 72K band to cdc7.4 by gel analysis of other cdc7 alleles.

Fig 34 shows the result of SDS polyacrylamide gel electrophoresis of whole cell extracts of some of the available alleles of cdc7.1, cdc7.3, cdc7.4 (H201.14.4) cdc7.4 (DE200.1.3), and cdc7.7. The 72K protein was present only in H201.14.4. However, this gel revealed a further set of differences in the region of 52.5K to 55K. Four and probably five bands were visible in various extracts, with considerable differences in intensity between cdc7.1, cdc7.3, cdc7.7 and H201.14.4.

Figure 33: DNA synthesis in cdc7.4 (DE200.1.3).

Experimental conditions were the same as for Figure 1, except that the cell number was 4.7×10^6 cells/ml at the start of the experiment, 4.4 μCi/ml 6-^{3}H uracil was used and the growth medium was YESD (+ amino acid supplements).

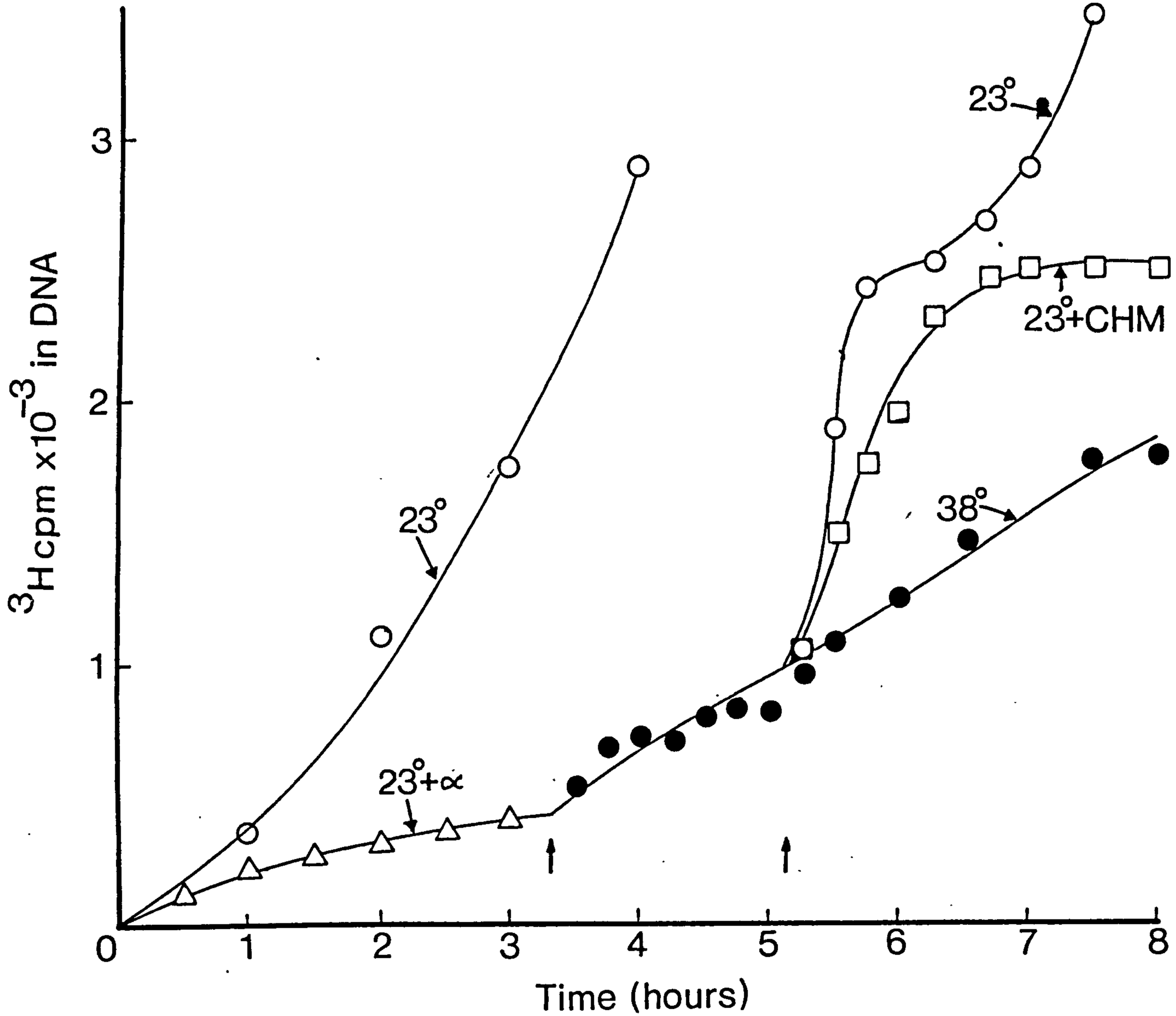
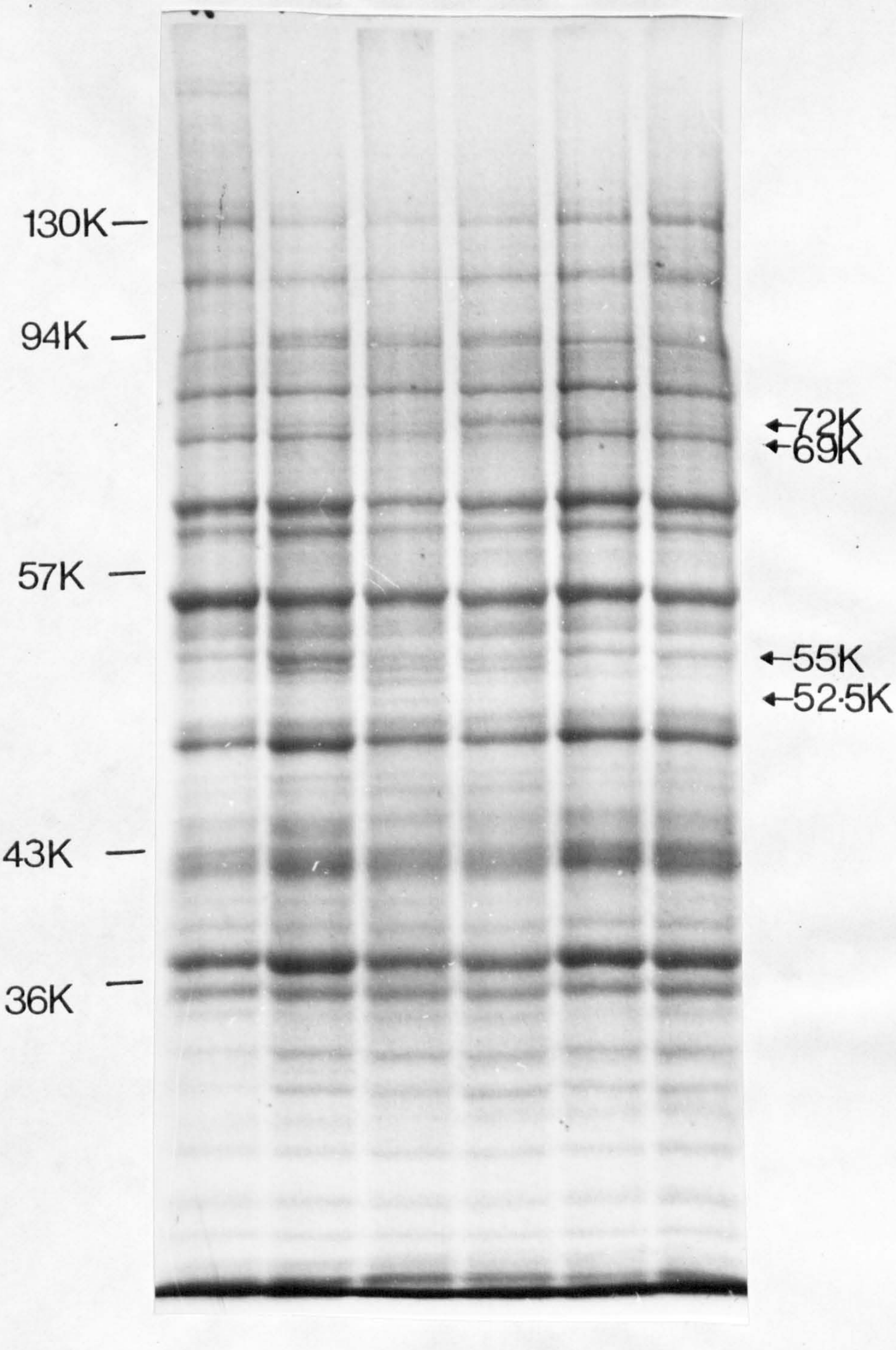

^{3}H cpm x10^{-3} in DNA
3
2
1
0
1
2
3
4
5
6
7
8
Time (hours)
23°
23°+CHM
38°
23°+α

Figure 34: SDS-polyacrylamide gel electrophoresis of whole cell extracts of various alleles of cdc7.

Whole cell extracts were prepared from 10ml cultures of cdc7.1, cdc7.3, cdc7.4 (H201.14.4), cdc7.4 (DE200.1.3), cdc7.7, and A364A, grown in YEPD-AU to 10^7 cells/ml as described in the legend to Figure 24.


130K
94K
57K
43K
36K
72K
69K
55K
52·5K
A364A
7.1
7.3
7.4
7.4
7.7
cdc
H201.14.4
DE200.1.3

The important comparison is now DE200.1.3. This showed a band pattern closely resembling A364A, and distinct from H201.14.4. Clearly the difference cannot be due to the defect in DNA synthesis, as it would be carried by DE200.1.3. The abnormal band pattern has been eliminated in the changed genetic background.

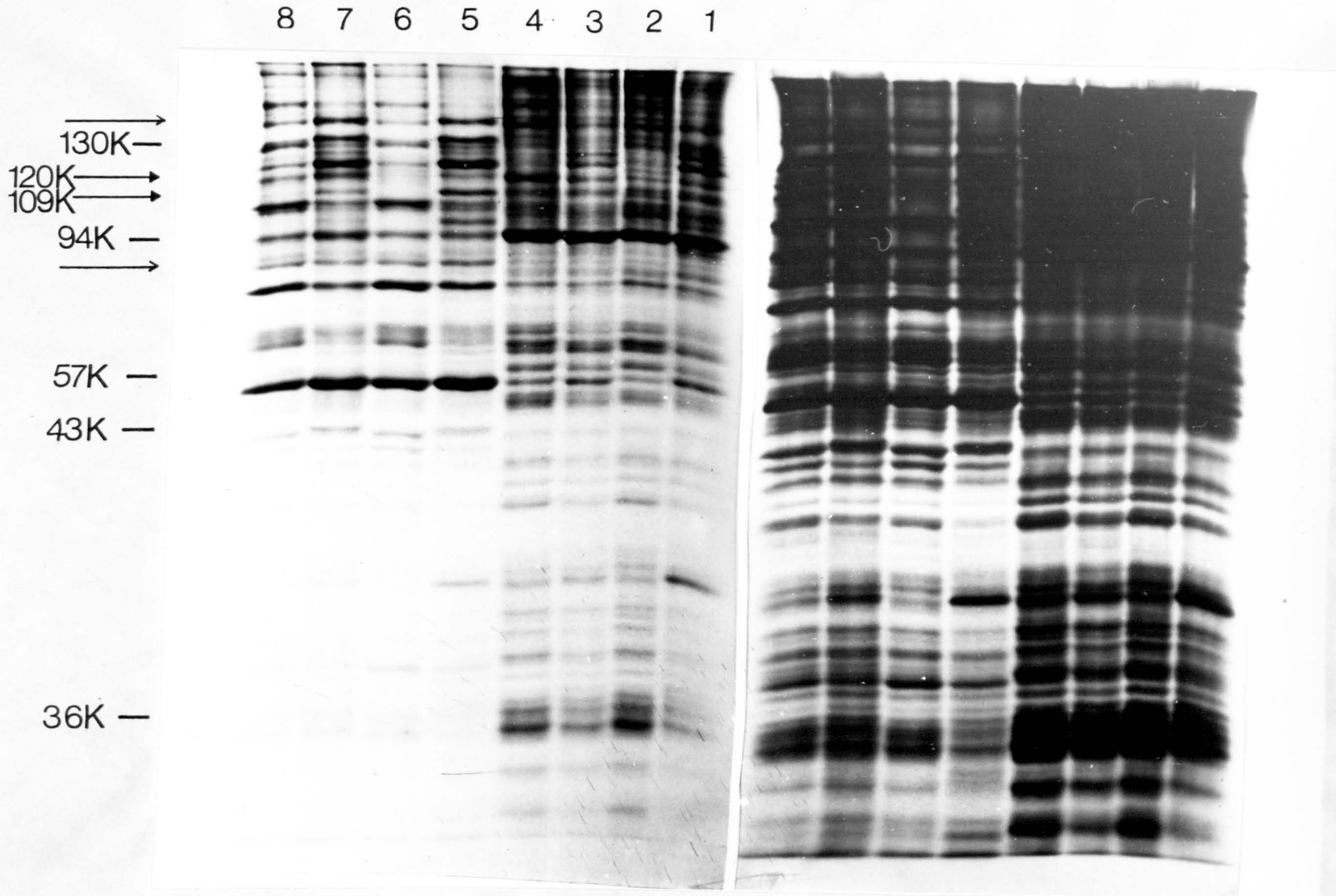
4.3.2.6. Analysis of nuclear and cyloplasmic proteins of cdc7.4. (DE200.1.3.).

Using the method described in 3.3.5., nuclear and cyloplasmic fractions were prepared from cultures of A364A and cdc7.4 (DE200.1.3.) which had been labelled with ^{14}C protein hydrolysate at 23°C and 38°C for 2½ hours. Proteins were separated on a one-dimensional SDS-polyacrylamide gel, which was then fluorographed (Fig 35). This figure contains two exposures of the same fluorograph, to enhance visualization of the heavy and light bands. The resolution on this gel was significantly improved compared to gels of whole cell extracts.

The temperature dependent band labelling changes were shown most markedly by the nuclear proteins. The briefly exposed fluorograph was obtained at a later date. This showed a band variation which had not been obvious in the overexposed copy. A band at 120KMW was depleted in A364A nuclei at 38°C (track 6), relative to A364A nuclei at 23°C (track 5) and cdc7.4 nuclei at 23°C and 38°C (tracks 7 and 8). There appeared to be a reciprocal relationship between this band and a smaller protein of 109KMW, because the intensities of the two were reversed in A364A and cdc7.4 nuclei both grown at 23°C, when compared with bands which had equal intensities in both tracks (arrowed). This may indicate a product-precursor relationship between the two bands. If this is the case, then the residual presence of the 120K protein in cdc7.4 nuclei at 38°C may be significant. Unfortunately, this information was not available at a time when the necessary checks for correspondence with cdc7.4 (H201.14.4) could have been made.

Figure 35: SDS-polyacrylamide gel electrophoresis of nuclear and cytoplasmic fractions from A364A and cdc7.4 (DE200.1.3).

90ml exponential cultures of cdc7.4 and A364A in $SD-AU_{10}$ at $8x10^6$ cells/ml were prepared as described in the legend to Figure 16. The cultures were split into two 45ml portions, 0.5 μCi/ml ($U-^{14}C$) protein hydrolysate was added, and one portion of each culture was shifted to 38°C. After a 2.5 hour labelling period, the cultures were given a 30 minute chase using 30mg/ml Casamino acids (Difco). The s and p_2 (cytoplasmic and nuclear) fractions were obtained as shown in Figure 17. Samples containing 5000 cpm were electrophoresed in a 10% SDS-polyacrylamide gel, which was then fluorographed (4.2.1.). Two exposures (2 days and 7 days) are shown. Bands at 120K and 109K MW are identified by arrows,[→]



Temp.°C	38	23	38	23	38	23	38	23
Strain	cdc7.4		A364A		cdc7.4		A364A	
Fraction	P_2				S			

as opposite

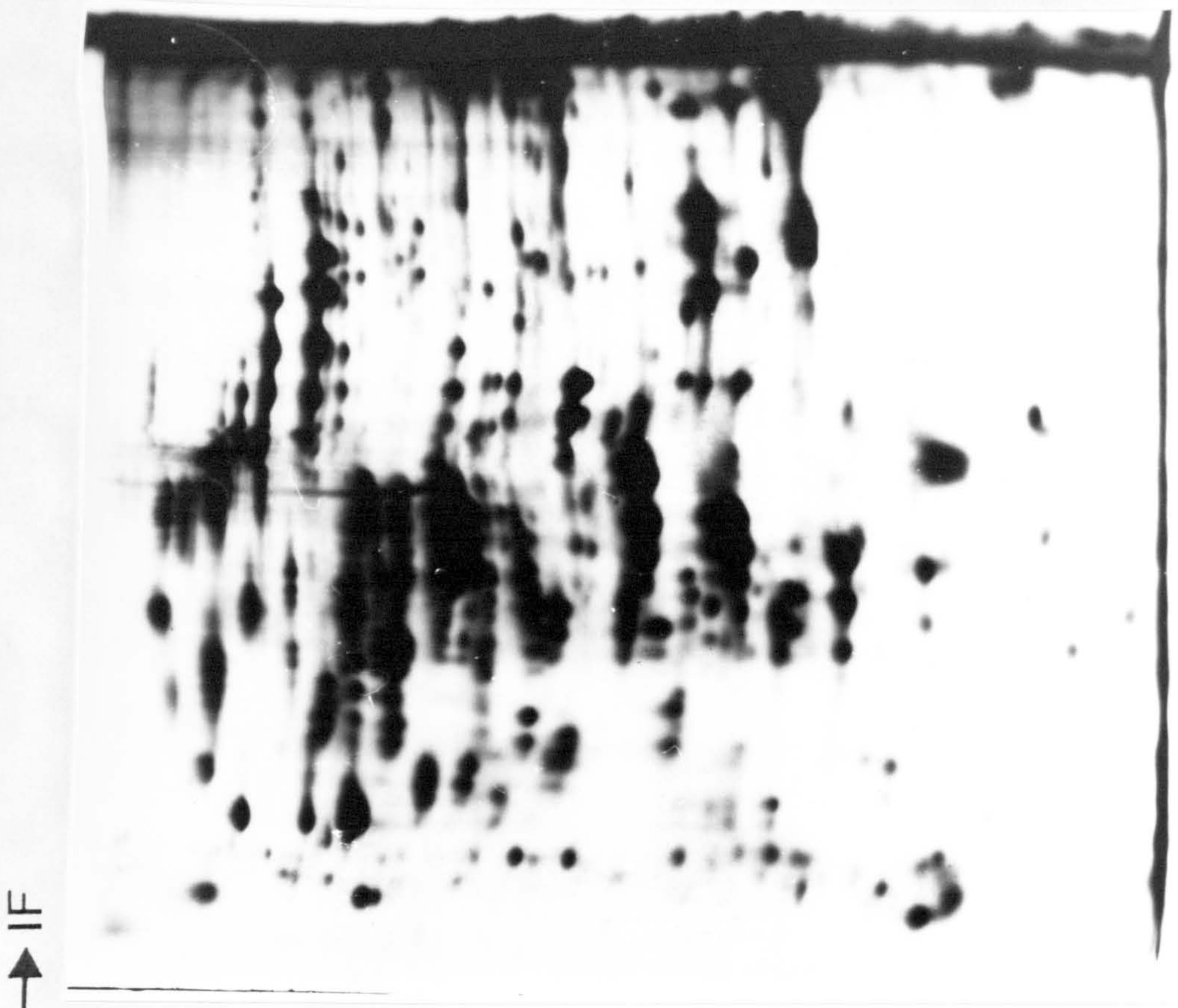
4.3.3. Two Dimensional Gel Analysis.

Cultures of A364A, cdc7.1, cdc7.4 (H201.14.4), cdc7.4 (DE200.1.3.) and cdc7.7 were labelled for 1 generation at 23°C (Fig 36). The major observable differences, and there were several in the region around pH6, and MW 45 - 57K, were between DE200.1.3. and H201.14.4. Other strains showed greater similarity with the latter. Under the conditions of exponential growth used here, it was not possible to detect significant differences between H201.14.4. and A364A. The 72K protein in cdc7.4 (H201.14.4) cannot be identified, so presumably it has a pI >7. Clearly this analysis requires expansion of the pH scale to include basic proteins.

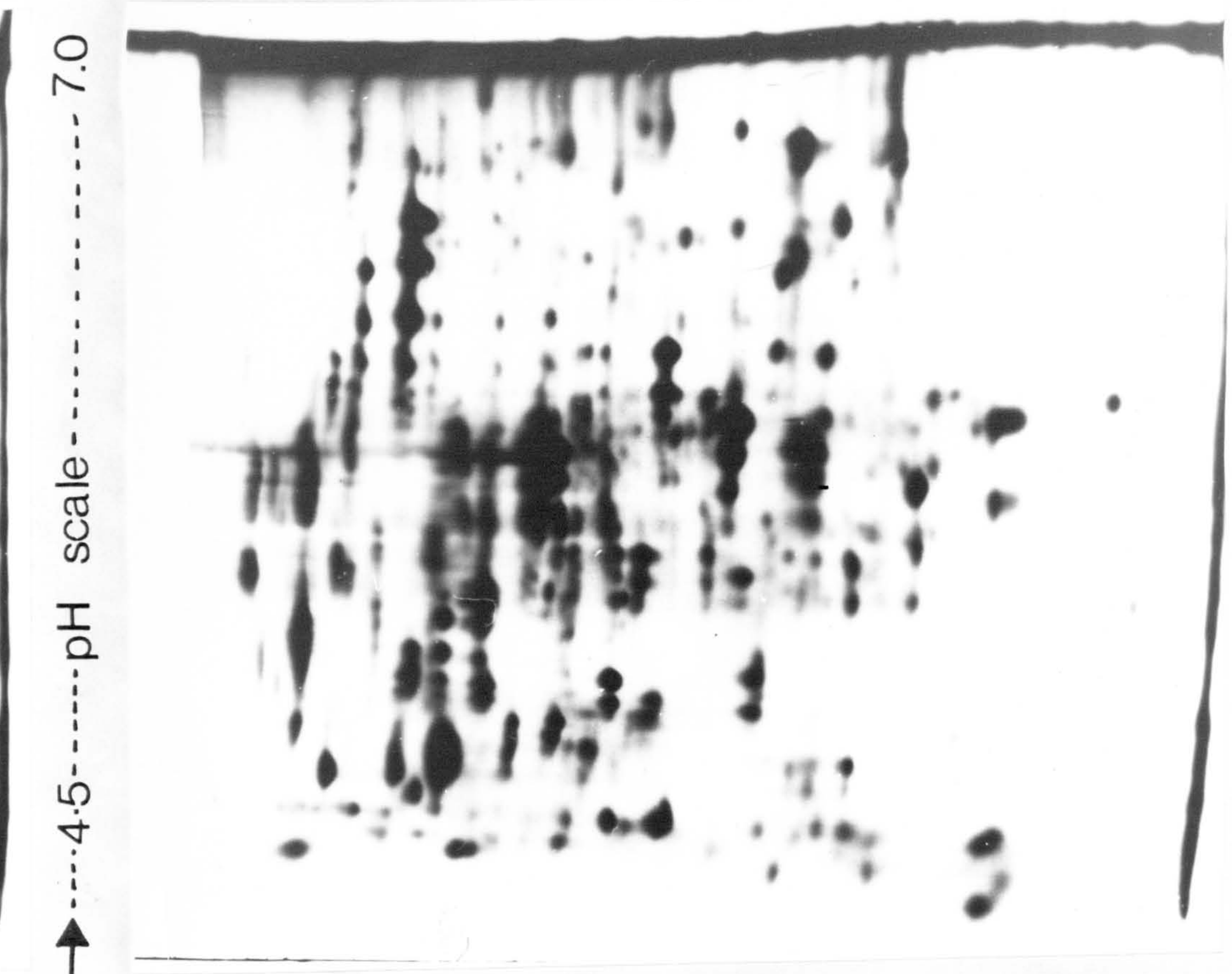
Figure 36: 2 dimensional polyacrylamide gel patterns of the alleles of cdc7.

Whole cell extracts were prepared from cultures of cdc7.1, cdc7.4 (H201.14.4) cdc7.4, DE200.1.3 and cdc7.7, which had been labelled using (U-^{14}C) protein hydrolysate as in Figure 24. Samples were prepared and electrophoresed as described in 4.2.1 and volumes equivalent to 20,000cpm were used for each gel. A, cdc7.1; B,cdc7.7; C,cdc7.4 (H201.14.4), D,cdc7.4 (DE200.1.3).

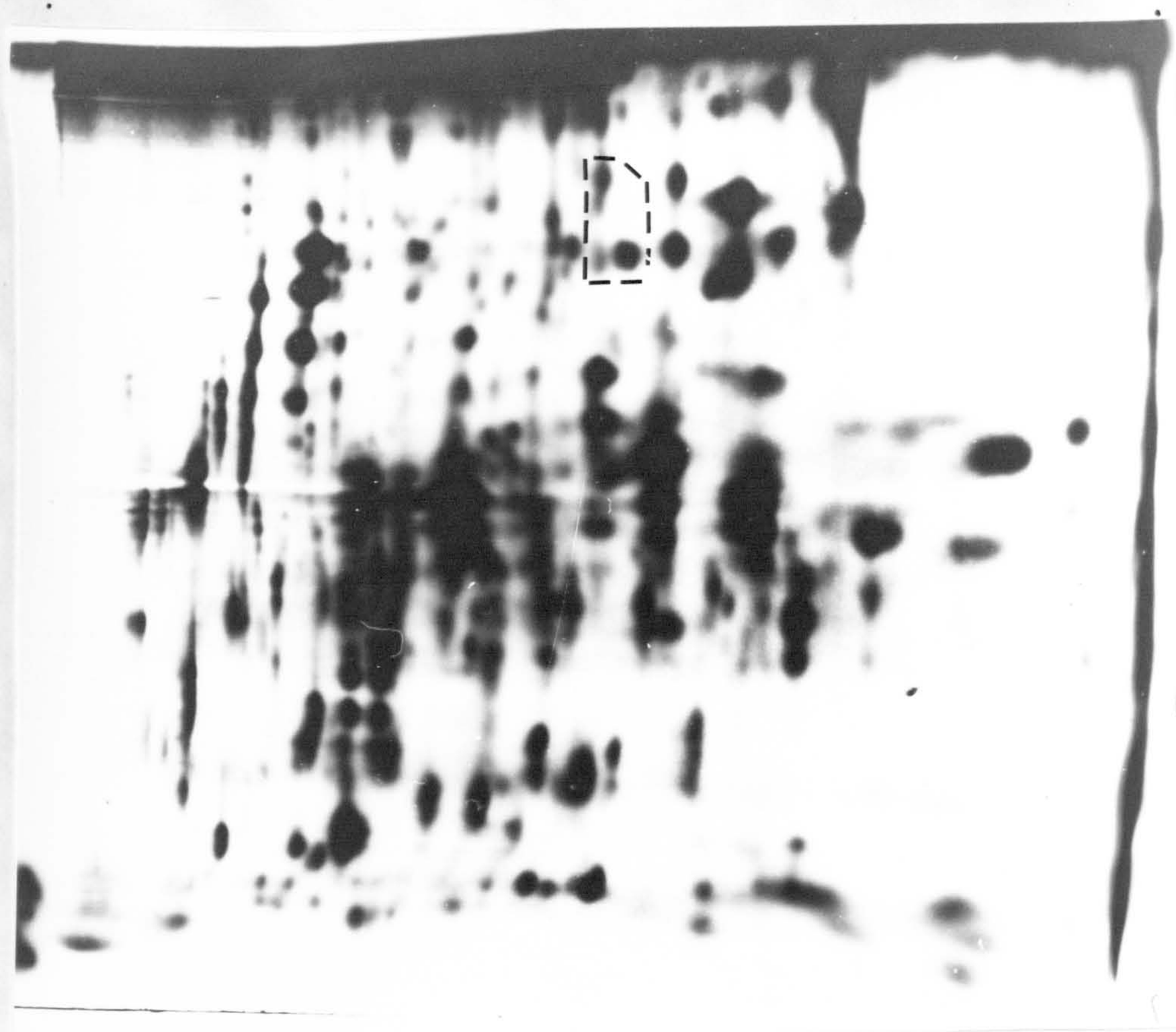
IF signifies the first, isoelectric focussing dimension; SDS, the second dimension. A region of heterogeneity is marked with dotted lines.



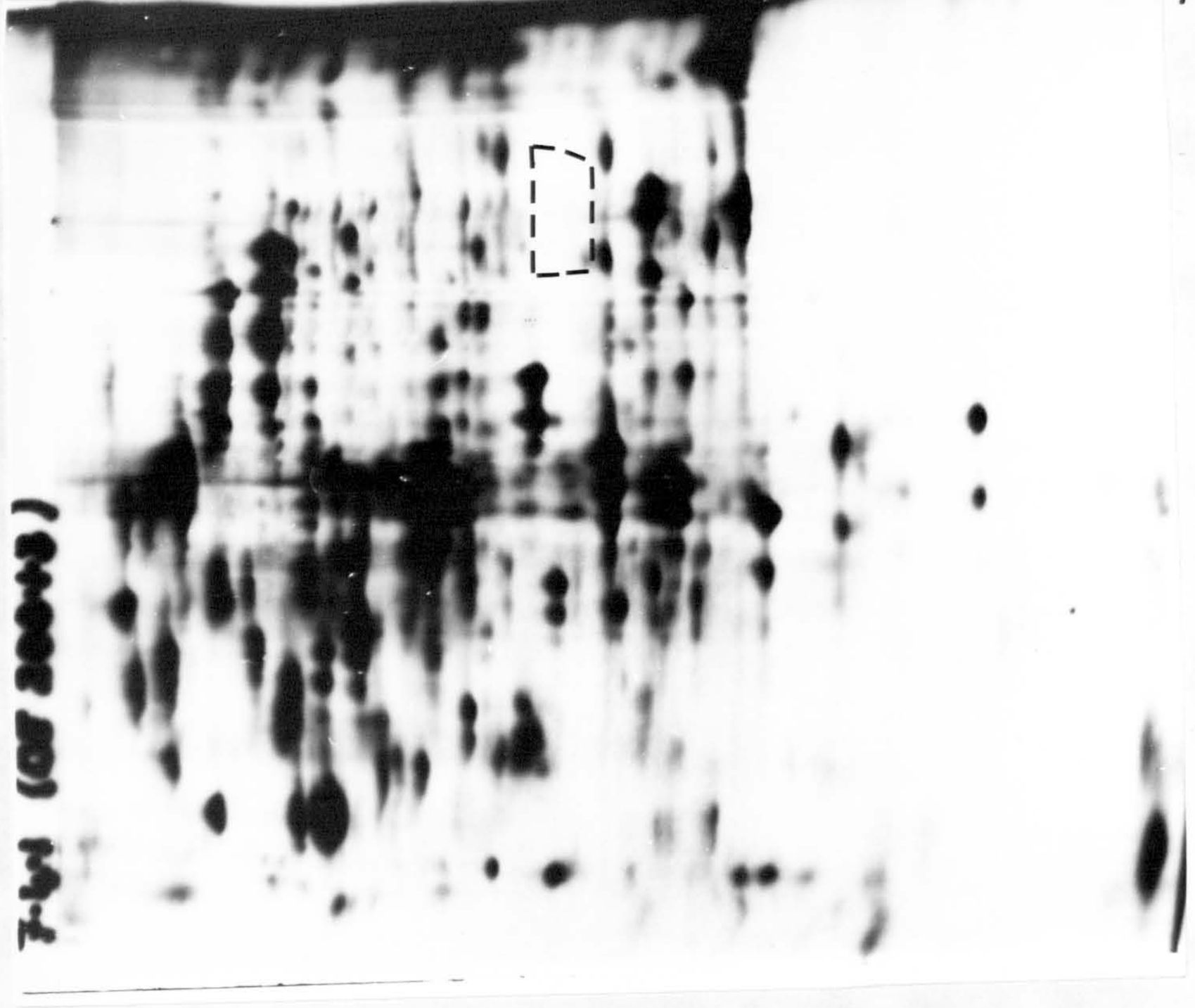
A; cdc 7.1



B; cdc 7.7



C; cde 7.4 H201.14.4



D; cde 7.4 DE200.1.3

4.4. CONCLUSION.

The anomalous protein at 72K discovered in cdc7.4 (H201.14.4) was not related to the defect in the initiation of DNA synthesis. The two characters segregate independently in genetic crosses. The protein was also not related to the presence of a second temperature-sensitive lesion, which was masked by the first in cdc7.4. Thus H201.14.4 was found to contain at least 3 completely independent differences from its wild-type parent. Pringle (1975) points out that induction of mutation by N-methyl-nitro-N-nitroguanidine (NNG) as used by Hartwell (1967), often results in multiple mutations within a short stretch of the chromosome. So it is possible that there are yet more mutations resident in H201.14.4. These may be the cause of the further abnormal bands seen in Fig 34. For this reason ethylmethanesulphonate (EMS) as a mutagen is preferable since it does not give multiple lesions, (Pringle 1975) and was used to generate cdc7.7, (Hartwell et al, 1973). This strain may prove more amenable to analysis.

The 72K band defect is interesting in its own right, since it appears to be related to a 69K protein in wild-type cells. The 72K protein does not appear to impart any biological impairment, and equal amounts of 72K and 69K proteins are made in a diploid strain (DE100). There may be a precursor-product relationship between these two bands, since many secretory proteins in higher enkaryotic cells are made as preproteins, from which a 1000-2000 MW signal peptide is later removed (Campbell and Blobel 1976; Geisow 1978). If this were the case, it could be argued that mutation has modified the cleavage site in the 72K preprotein. Alternatively, the 72K protein may result from mutation of a termination codon, allowing read through.

The ts2 lesion carried by H201.14.4 confers an inability to grow at 38°-39°C. The evidence suggests that this mutation allows multiple rounds of cell division before arresting. Hartwell (1974) discussed such mutants and concluded that they may result from defective folding

during synthesis of an altered protein at high temperature. Proteins synthesised at lower temperatures adopt an active conformation which is retained on raising the temperature. Therefore the cell can continue growing and dividing at the restrictive temperature until the activity is diluted out.

One-dimensional gel electrophoresis and analysis of ^{32}P-phosphate labelled proteins did not shed any light on potential mutant proteins. It was hoped that the extra resolving power of 2D-gel electrophoresis would provide an answer. West and Emmerson (1977) had used this technique to successfully identify protein X of E.coli as the recA gene product, through the change in mobility of the protein from a tif-1 (recA) mutant. However, the problems encountered were unexpected. The strains obtained from L. H. Hartwell were reported to be isogenic (Hartwell et al 1973), but differences in their protein profiles were observed in one-dimensional gels. This may be due to the presence of additional undetected mutations, such as those found here in cdc7.4 (H201.14.4). The patterns on 2-D gels were also found to be dependent on the genetic background of the strains. The outcrossed strain DE200.1.3 showed several differences from the original H201.14.4, which cannot be related to the lesion in DNA synthesis. The technique is undoubtedly a sophisticated one capable theoretically of resolving the gene products ($\approx$ 6000) of the entire E.coli genome (O'Farrell 1975). In the limited analysis performed here, it did not provide a solution.

Many proteins were observed on SDS-polyacrylamide gels whose synthesis varied dramatically with changes in the growth temperature of the cells. This effect had not been reported previously. However, it proved to be a major problem in the search for a mutant protein using radioactive labelling at the restrictive and permissive temperatures.

The nuclear preparation characterised earlier (3.3.5) clearly

gave an enhancement of nucleus specific proteins. It allowed the detection of an abnormal band pattern in cdc7.4, not observable on SDS-polyacrylamide gels of whole cell extracts. The approximately equal intensities shown by several major and minor bands (arrowed in Figure 35) which do not vary between A364A and cdc7.4 but which depend on the growth temperature of the cells, suggests that the purification procedure does not result in selective loss of proteins, and that comparisons of bands which show variations between strains are justified.

CHAPTER FIVE

Speculations on possible defective functions in cdc7.4

CHAPTER FIVE.

SPECULATIONS ON POSSIBLE DEFECTIVE FUNCTIONS IN cdc7.4.

5.1. INTRODUCTION.

The crucial role of the CDC 7 gene product in the successful initiation of DNA replication (Chapter 3), allows speculation on possible defective functions. Indeed, as an adjunct to the search for a mutant protein, and the development of an *in vitro* DNA synthesising system, it was important to establish that DNA polymerase, RNA polymerase, and other assayable activities implicated in DNA replication (1.5.6.2), are not responsible for the defect.

Apart from abnormalities in these proteins, there are good grounds for suspecting that certain metabolic deficiencies might influence the initiation of replication. Cyclic nucleotides appear to fluctuate during the cell cycle (review, Friedman, 1976). Elevated levels of cAMP have been shown in inhibit serum-stimulated DNA replication in human fibroblasts (Rechler *et al*, 1977). Estimates of cGMP levels in proliferating and non-proliferating cells have strengthened the idea that cGMP has an opposite effect to that of cAMP, as elevated levels are associated with proliferation (Goldberg *et al*, 1974). However, Wang *et al* (1978) have shown that a rise *and* fall of internal cAMP levels is necessary for the entry of lymphocytes into S phase. The effects of these and other nucleotides upon growth of the cell cycle mutants at the restrictive and permissive temperatures were observed on nutrient agar plates.

Rapaport and Zamecnik (1976) reported the presence of diadenosine $5^1, 5^{111}$- P^1, P^4- tetraphosphate (Ap4A) in various eukaryotic cell lines, the levels varying dramatically with the proliferative activity of the cells. Inhibition of protein synthesis, or the arrest of proliferation by serum deprivation reduced the level of Ap4A up to 50 fold. Grummt (1978a,b) reported that Ap4A triggered DNA

replication in permeabilized G1 arrested BHK cells. However, the product characterisation was limited to the demonstration of replication eyes in 2.8% of molecules observed by electron microscopy. The specific binding of Ap4A to DNA polymerase-α and to no other purified DNA polymerase has been alleged and suggests an effect on nuclear DNA replication. However, the significance of this work remains to be established. An analysis of the effects of the nucleotides Ap4A and Gp4G on the cell cycle mutants was not undertaken in this work, but forms part of another thesis from this laboratory (F.Z. Watts).

Modification of purine nucleotides in DNA by methylation or glucosylation is a widespread phenomenon. In bacteria, there are numerous distinct methylase activities, including an adenine methylase (dam gene product), a cytosine methylase (dcm gene product), and the methylases of the Type I and Type II restriction-modification systems (for review, Kornberg, 1980). The restriction-modification methylases represent the method by which the cell distinguishes foreign DNA from its own. The roles of the other methylases are unresolved, but the hyperecombinational behaviour of dam-mutants suggests that dam-mediated methylation may regulate recombination (Konrad, 1977). It has been known for some time that the substitution of ethionine for methionine in the growth medium of methionine auxotrophic bacteria allows completion of rounds of replication in progress, and initiation of further rounds, but replication stops abruptly when the region of non-methylated DNA comes to be replicated again (Lark and Arbor, 1970). Lark (1979) reported that the DNA synthesising ability of lysates of E. coli cells which had been grown in the presence of ethionine, could be restored by the addition of SAM and ATP, and that this synthesis, which did not appear to be repair, was performed by DNA polymerase I. However, the relevance of this phenomenon to the in vivo condition must be questioned, since ts polA strains restart DNA replication upon addition of methionine at both permissive and restrictive

temperatures. The fact remains that the correct methylation of DNA is a prerequisite for its replication.

Restriction-modification enzymes in prokaryotes fall into two categories, TypeI and TypeII. (Boyer, 1974). TypeI enzymes, e.g. Eco B and Eco K, are structurally complex, have requirements for Mg^{2+}, SAM and ATP, and cleave away from their recognition sites (Linn et al, 1974, Yuan et al, 1975). Type II enzymes such as EcoRI require only Mg^{2+} for activity, and cleave at their recognition sites (Boyer, 1974).

Together with its role in phosphatidyl choline biosynthesis from phosphatidyl ethanolamine (Lehninger, 1970), this knowledge of the involvement of SAM in the recognition and usage of DNA sequences by proteins in bacteria prompted a study of the effect of exogenous SAM upon growth of the cdc mutants. In S.cerevisiae, the pool of SAM is labelled very rapidly (20 seconds) when cells are supplied with ^{3}H-methionine (Warner et al, 1976). Thus it was possible that an SAM-dependent protein was in some way involved in the initiation of DNA synthesis in S.cerevisiae, and that the lesion in cdc 7.4 might be a defect in SAM synthesis, e.g. SAM synthetase. This could also explain the lack of viability of cdc7.4 at the restrictive temperature (3.3.1.1). The rapid burst of DNA synthesis seen on dropping arrested cdc7.4 cells to the permissive temperature is consistent with the SAM pool labelling reported by Warner et al (1976). A study of the levels of SAM synthetase in A364A and the available cdc7 strains was also undertaken.

A report by Quesney-Huneeus et al (1979) suggests a connection between 3-hydroxymethyl-3methylglutaryl (HMG) CoA reductase activity and DNA replication, since the enzyme was observed to increase in activity at or just prior to the peak of DNA replication in synchronised BHK cells. Compactin, a competitive inhibitor of HMG CoA reductase, completely abolishes S phase and this inhibition can be reversed by the addition of mevalonic acid, the product of the HMG CoA reductase

reaction. Mevalonate is a metabolite on the pathway to cholesterol biosynthesis (Lehninger, 1970). Thus as with the proposed SAM deficiency, a defect in mevalonic acid could account for the characteristics of cdc7.4. The value of such screening tests were confirmed by the work of Gaine (1976) who demonstrated that the mutation in cdc21.1 was probably due to a defective thymidylate synthetase by correcting the ts phenotype on agar plates supplemented with dTMP.

Strand separation is a logical necessity for the initiation of DNA replication. There are now several known cases in prokaryotes where this is achieved by the introduction of a nick in a specific region, yielding a 3^1OH group which serves as a primer for DNA replication (1.5). Henry and Knippers (1974) purified the gene A protein of bacteriophage ØX174 which performs this action at a specific site on the viral strand of ØX174 RFI DNA (Langeveld et al, 1978). Geisselsoder (1976) showed that gene A of bacteriophage P2 produced a strand specific discontinuity required for replication. The gene II protein of bacteriophage fd nicks supercoiled RFI fd DNA in the viral strand, and will not relax any other supercoiled DNA (Meyer and Geider, 1979). Such activities have not been discovered for bacterial chromosome and eukaryotic DNA initiation. In the mid-1970's, interest became focussed on nucleoprotein complexes, in which form many plasmids such as ColE1 and R factors could be isolated using gentle lysis techniques. They were termed "relaxation complexes", because treatment with protein denaturing reagents or conditions resulted in relaxation of the supercoiled plasmid by the introduction of a single nick and covalent attachment of protein to the DNA (Blair and Helinski, 1975). The relaxation event in ColE1 was found to be strand and site specific, 19% of a genome length away from the single EcoR1 site (Lovett et al, 1974a). The fact that this site could not at first be distinguished from the replication origin (Lovett et al, 1974b), and

the apparent similarity with a bona fide initiation protein, ØX174 gene A protein, in the attachment of protein to the 5^1 terminus of the nicked DNA strand (Guiney and Helinski, 1975), suggested at that time that these complexes were involved in the initiation of plasmid DNA replication. Similar nicked DNA-protein complexes were isolated from SV40 (Kasamatsu and Wu, 1976) indicating that this was not an exclusively prokaryotic phenomenon.

It was therefore of interest to assay the temperature-sensitivity of endonucleases from cdc7.4. The supercoiled covalently closed circular yeast 2 μm plasmid was an ideal substrate for such assays, but it was not known at that time whether its replication was dependent on the function of the CDC gene products. This was a crucial point to establish, since it had already been shown that mitochondrial DNA replication was unaffected by the cdc28, 4 and 7 mutations (3.3.2.2.). Experiments were undertaken to resolve this issue.

The use of the 2 μm plasmid in the endonuclease assay necessitated its preparation in quantity, and radioactively labelled. Several methods were assessed, and the most successful is described in detail here. At a later stage in the work, a recombinant plasmid became available containing the entire yeast 2 μm plasmid. Beggs (1978) reported the isolation of several hybrid plasmids, one of which, pJDB219, consisted of the yeast 2 m plasmid sequence, a ColE1 derivative pMB9, and a yeast chromosomal leu 2 sequence. The plasmid can be propagated in suitable strains of both E.coli and S.cerevisiae. It has the advantage of ease of purification from bacteria in considerably greater quantities than the yeast 2 μm plasmid had ever been obtained from S.cerevisiae. Also, since it contains the full 2μm plasmid sequence, any putative sequence specific activity should be equally evident using pJDB219.

Four endonuclease activities have so far been reported for

S.cerevisiae. Pinon (1970) described endonuclease A, which requires Mg^{2+} or Mn^{2+}, and has a 750 fold preference for single-stranded DNA over double-stranded DNA. Two endonucleases, B and C, both inactive on denatured DNA have also been separated (Pinon and Leney, 1975). They were distinguished by the requirement of endonuclease B for Mn^{2+}, and the action of endonuclease C in making single strand nicks with Mg^{2+} as cofactor, but double strand breaks with Mn^{2+}. Bryant and Haynes (1978) isolated an endonuclease from nuclei of S.cerevisiae which also functioned preferentially on denatured DNA. This enzyme was thought to be distinct from endonuclease A (Pinon, 1970) due to its ability to insert random nicks into ØX174 RFI DNA, and hence it was designated endonuclease α. The in vivo functions of these enzymes have not yet been established.

METHODS

5.2.1. Additions to growth media.

10^6 cells were grown on YEPD-AU agar plates at 38°C and 23°C in the presence or absence of 100 μM cGMP, or 1mM cAMP (Town et al, 1976), NAD, NADH, SAM, or mevalonic acid (MA). Plates were scored after 3-4 days.

5.2.2. RNA polymerase separation.

Assay of RNA polymerase activity was performed as in 2.13.1. Cell extracts were obtained from cells grown in YEPD-AU to 1-2x10^8 cells per ml (mid-log phase). 10g wet weight cdc7.4 (H201.14.4.) cells were mixed with 20ml. Extraction Buffer (0.2M Tris-HCl pH 7.9, 20% glycerol 20mM $MgCl_2$, 0.8M$(NH_4)_2SO_4$, 1mM EDTA, 1mM DTT, 3.4mM PMSF), and homogenised by 3x20 second bursts in a Braun homogeniser (Botany Dept. UCL). The beads were washed with extraction buffer and the extract (35ml.) spun at 46K rpm in a Beckman 50Ti rotor for 90 minutes at 4°C. The Spinco supernatant (25ml.) was diluted to 200ml. and loaded onto a DEAE-Sephadex A25 column (2x32.5 cm.) equilibrated with 0.05M Ammonium sulphate in 50mM Tris-HCl pH7.9, 25% v/v glycerol, 0.5mM EDTA, 1mM DTT (TGED) containing 1.7mM PMSF. The column was washed with 100ml of this buffer then eluted with a 700ml linear 0.05-0.45M Ammonium sulphate gradient.

5.2.3. Isolation of dTMP permeable (tup)mutants.

CAD agar plates (2.3) containing aminopterin, sulphanilamide and dTMP were used. The components of CAD were dissolved in 400ml distilled water and autoclaved. 600ml of a solution containing 4g sulphanilamide and 20mg aminopterin (dissolved in 1ml DMSO) was sterile filtered and added when cold. A sterile filtered solution of 10mg/ml dTMP was added to each agar plate to give a final concentration of 100μg/ml.

10^7 cells from a stationary phase culture of cdc21 (H146.2.3) were plated out and incubated at 23°C for 10 days. Approximately 15 colonies/plate appeared. The biggest of these were picked off, and screened for ability to incorporate ^{3}H dTMP into DNA.

5.2.4. Purification of yeast 2 μm plasmid on Benzoylated-naphthoylated-DEAE (BND)-cellulose.

Cells from 2 litres of mid-log phase cultures were spheroplasted using Arthrobacter enzyme (2.9). After washing by centrifugation through 1.5M sorbitol 0.1M EDTA, the spheroplasts were resuspended in 0.1M Tris-HCl pH7.6, 0.1M EDTA (TE buffer), and lysed by addition of sarkosyl L-35 to a final 3%. The lysate was maintained at room temperature for 45 mins, then spun at 27K rpm in a Beckman 50Ti rotor at 4°C for 30 minutes. The supernatant was decanted into a sterile measuring cylinder, and solid NaCl was added to 1M final. After dissolving, PEG 6000 was added to 10% w/v, and the extract was transferred to a sterile 50ml glass centrifuge tube and kept on ice for at least 2 hours. The PEG precipitate was removed by centrifugation at 1000 rpm in a MSE bench centrifuge for 5 minutes at 4°C, and resuspended in 0.1M TE buffer pH7.6. One-tenth volume of 5mg/ml boiled RNAaseA was added, and the mixture dialysed overnight against 1 litre 0.01M TE pH7.6. Proteinase K was added to 75 g/ml and incubated at 45°C for 2 hours. One-ninth volume of 3M NaAcetate pH6.0 was added, followed by 2 volumes of chilled absolute alcohol, and the DNA precipitated at -70°C for 2 hours.

The DNA precipitate was collected by centrifugation at 3K rpm in an MSE bench centrifuge for 10 minutes at 4°C, and resuspended in 0.001M TE pH8.0. After complete rehydration, 1M NaCl was added to a final concentration of 0.3M. Using a Type CMAW72 pH electrode, (Russell pH Ltd.), the pH was adjusted to 11.85 at 20°C by addition of 0.5N NaOH. After 3 minutes at this pH, N HCl/M Tris base (9/1) was added to bring the pH down to 8.0 at which point the preparation was

chilled in ice.

BND-cellulose (Boehringer; recycled by washing twice in 6M guanidinium chloride (GuCl) and twice in sterile 0.1M TE pH7.6, then with several volumes of 0.3M NaCl 0.001M TE pH7.6) was packed hard into a 2cm diameter column to give a bed height of 1.8cm. The extract was loaded onto the column, followed by 30ml 0.3M NaCl 0.001M TE pH7.6. Plasmid DNA was eluted with 1M NaCl 0.001M TE pH7.6, then ethanol precipitated as before. Single-stranded DNA was eluted with 6M guanidinium chloride (Aristar, BDH).

5.2.5. Preparation of ^{3}H-ColE1 DNA and ^{3}H-pJDB219 DNA.

50ml cultures of DS581 ColE1^{+} E.coli (str-r, thy^{-}) in L-Broth or M-9 medium (2.3) were labelled overnight at 37°C with 2 μCi/ml 6-^{3}H thymidine (2.1). If amplification was required, 2.5mg/ml chloramphenicol (Sigma) was added (sterile filtered) to a final concentration of 100 μg/ml. Plasmid was prepared as described in 2.16

pJDB219 was prepared similarly from the E.coli strain JA221 (recA1, le B6, trp E5, sdR^{-} sdM^{+} lacY C600; Beggs, 1978). Tetracycline was sterile filtered into the medium (L-Broth) to a final concentration 15μg/ml. All operations with this organism and the plasmid pJDB219 were performed under category P1 containment conditions.

5.2.6. Nitrocellulose filter binding endonuclease assay.

The method was that of Center et al, (1970). Reaction mixtures (0.2ml) contained 50mM Tris-HCl pH8.0, 7mM $MgCl_2$, 15 μgBSA (from a 0.5mg/ml stock solution, heated at 75°C for 45 minutes to destroy nucleases), 1.5mM DTT, 0.2-0.4 μg plasmid DNA (700-2000 cpm).

0.1ml diluted enzyme and 0.1ml concentrated mix were preincubated separately in sterile glass tubes for 45 seconds at the raction temperatures before mixing and reacting for 3-5 minutes. Reactions were terminated by the addition of 0.1ml 0.1M Tris-HCl, pH8.0, 0.1MEDTA, followed by 1ml 1xSSC (0.15M NaCl, 0.015M sodium citrate, pH7.0).

Mixtures were boiled for 3 minutes, then chilled immediately in ice. 5ml ice-cold 6xSSC was added and the mixtures were filtered slowly (2ml/minute) through nitrocellulose Millipore filters HAWP 02500. Filters were washed with an additional 5ml 6xSSC, dried, and counted in PPO-POPOP-toluene scintillation fluid (2.6.4).

5.2.7. Preparation of crude extracts for fractionation of endonuclease activities.

10-20g wet weight of cdc7.4 (DE200.1.3) cells grown to mid-log phase in YEPD medium (2.3) were harvested and broken in a Braun homogenizer as described in Section 2,[12] except that 2M NaCl was omitted from the extraction buffer. After washing the glass beads, solid NaCl was added to 1M, and the extract was clarified by centrifugation at 38K rpm for 1 hour at 4°C in a Beckman 50Ti rotor. The supernatant was dialysed for 5 hours (4 changes) against 2 litres TEDP buffer pH7.5 at 4°C, then centrifuged at 15K rpm for 10 minutes at 4°C in a MSE 18 centrifuge. The extract was then loaded onto DEAE-cellulose columns as described in the legends to Figures 43 and 44.

5.3 RESULTS AND DISCUSSION.

5.3.1. Effect of addition of metabolites upon growth.

The effects of addition of cAMP, cGMP, NAD, NAD(H), S-adenosylmethionine, and mevalonic acid upon growth of A364A, cdc4, cdc7 and cdc28 on nutrient agar plates (5.2.1) are summarised in Table VIII. No compound restored growth of any of the mutants at 37°C. Growth was unaffected at 23°C. Colony forming ability at 37°C of the parent strain A364A was equal to that at 23°C(+++), but colony size was markedly reduced(+).

Table VIII. Effect of additions upon growth.

Addition	Growth.							
	A364A		cdc4.3		cdc7.4		cdc28.1	
	23°	37°	23°	37°	23°	37°	23°	37°
none	+++	+	+++	-	+++	-	+++	-
cAMP	+++	+	+++	-	+++	-	+++	-
cGMP	+++	+	+++	-	+++	-	+++	-
NAD	+++	+	+++	-	+++	-	+++	-
NAD(H)	+++	+	nt	nt	+++	-	nt	nt
SAM	+++	+	+++	-	+++	-	nt	nt
MA[2]	+++	+	nt	nt	+++	-	nt	nt

Notes:

1: All additions were 1mM final except for cGMP which was 100 µM.

2: cdc7.4 strain was DE200.1.3; all others were H201.14.4 (Section 2.2).

SAM synthetase activity was assayed in crude extracts of A364A and the collection of cdc7 mutant alleles using the method of Holcomb and Shapiro (1975). Surprisingly, levels of the enzymes were found to vary by as much as six-fold between strains, whereas DNA polymerase and alcohol dehydrogenase specific activities were fairly constant. However, SAM synthetase activity was greater in all the strains tested when measured at 37°C than at 23°C. (This work was performed in conjunction with I. R. Johnston).

5.3.2. Thermosensitivity of RNA polymerases from cdc7.4.

RNA polymerases I, II and III were separated on DEAE-Sephadex A25 by the method of Shultz and Hall (1976); 5.2.2. Fractions were assayed at 23°C and 36°C as described in 2.13.1. The results are shown in Fig. 37. Simultaneously, fractions were assayed for their sensitivity to 20 μg/ml α-amanitin, the results suggesting that the ascribed order of elution as being polymerase I, II and III was correct. In yeast, RNA polymerase III is resistant to 2 mg/ml α-amanitin, whereas polymerases II and I can be inhibited by 20 μg/ml and 2mg/ml respectively (Valenzuela et al, 1978). In this fractionation, polymerase III was incompletely separated from polymerase II, appearing as a shoulder on the trailing edge of the polymerase II peak. This was probably an artifact of the extraction and chromatographic procedures used, since an A364A extract shows a similar profile (data not shown).

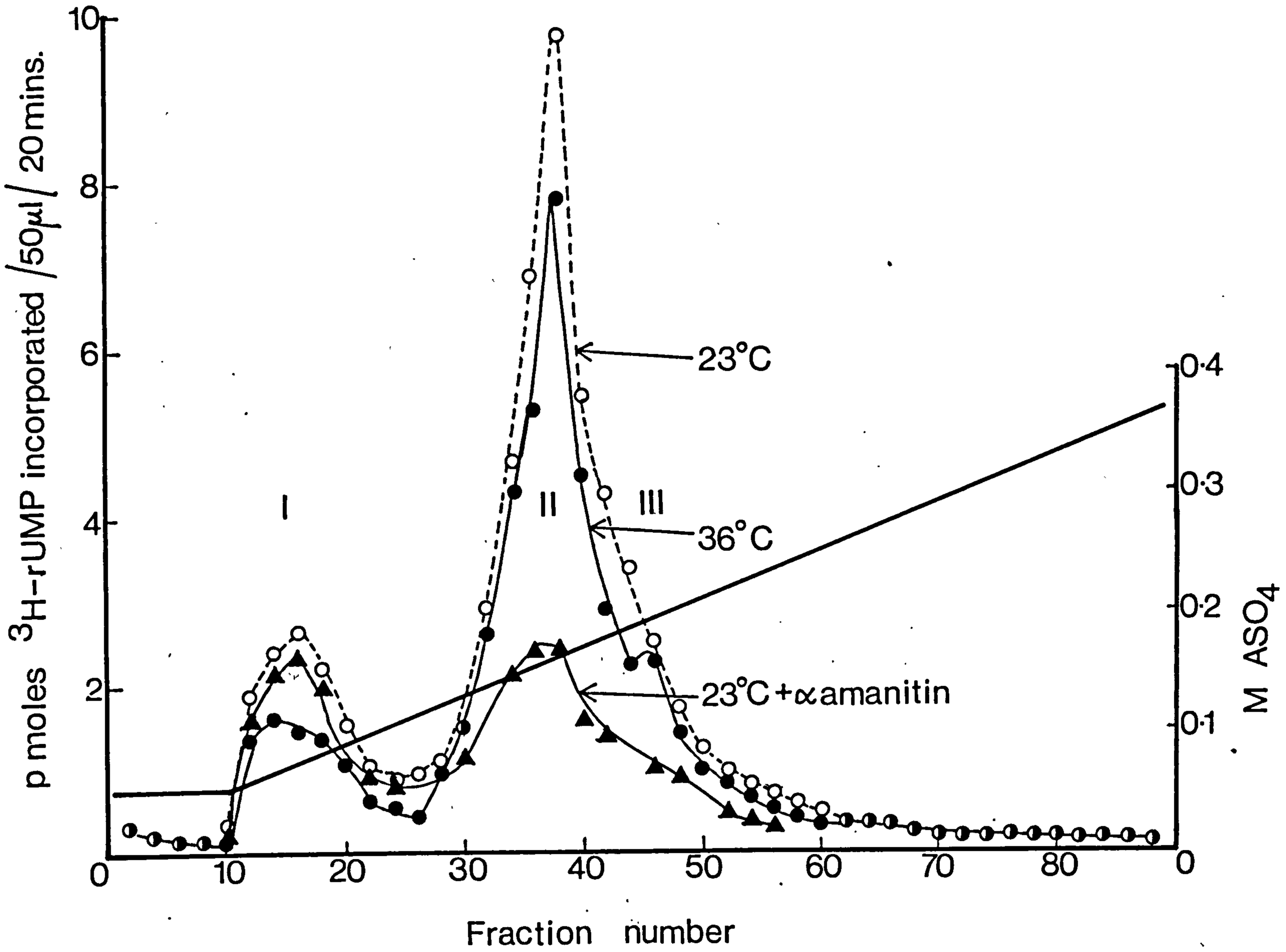
There was no obvious temperature-sensitivity of any species of RNA polymerase using this assay. This does not eliminate the possibility that the cdc7 mutation might affect a regulatory subunit equivalent to E.coli RNA polymerase σ factor, (Chamberlin, 1974), or the Ngene product of bacteriophage λ (Lewin, 1977) which would not be detected.

5.3.3. Thermosensitivity of DNA polymerase and single-stranded DNA dependent ATPase activities from cdc7.4 and A364A.

Crude extracts obtained from log phase A364A and cdc7.4 cells (5.2.2) were assayed for DNA polymerase activity (2.14) at varying dilutions for 15 minutes at both 23°C and 37°C. At equivalent protein levels (approximately 200μg protein/assay) the incorporation into acid precipitable material at 23°C corresponded to 224 and 266 nmoles total nucleotide for A364A and cdc7.4 respectively, and at 37°C the equivalent values were 697 and 798 nmoles respectively. Thus there was no

Figure 37: Temperature sensitivity of RNA polymerases from cdc7.4.

RNA polymerases from log-phase cdc7.4 (H201.14.4) cells were separated on a 2.0x32.5cm column of DEAE-Sephadex A25 as described in 5.2.2. 20 minute assays were performed at 23°C (O), 36°C (●) and at 23°C in the presence of 20 µg/ml α-amanitin (▲) as described in 2.13.1.


p moles ^{3}H-rUMP incorporated /50µl/ 20 mins.
M ASO_4
Fraction number
I
II
III
23°C
36°C
23°C +α amanitin
0
2
4
6
8
10
0·1
0·2
0·3
0·4
0
10
20
30
40
50
60
70
80

detectable thermosensitivity by this assay, which is in keeping with the initiation phenotype of cdc 7.4 (see section 6.3.4.1 for DNA polymerase separately).

Using the assay for DNA-dependent ATPase activity described by Assairi and Johnston (1979), it was established that when the extracts used above were fractionated by chromatography on phosphocellulose P11, neither the column flow-through or the activity peak eluted with a 0.25M potassium phosphate wash showed detectable thermosensitivity in assays ranging from 15 to 45 minutes. The results are presented in Table IX.

TABLE IX. DNA-dependent ATPase activities*

	A364A		cdc7.4	
	23°C	37°C	23°C	37°C
Phosphocellulose Flow-through	8.27	7.87	6.80	8.75
Eluted activity peak fraction	2.45	4.96	2.48	4.91

*Activity expressed as nmoles ADP produced in 30 minute reactions, separated on PEI-cellulose paper (Schleicher and Schull).

5.3.4. The Yeast 2μm plasmid.

5.3.4.1. Control of replication of the yeast 2μm plasmid.

The 2μm plasmid has the same density (ρ =1.699 gcm^{-3}) as nuclear DNA (Carter, 1975), but they can be separated using ethidium bromide-caesium chloride gradient centrifugation. The dependence of 2μmplasmid replication upon the CDC7 gene product was tested by determining whether the plasmid was replicated in cultures arrested at the cdc7 block. This was done using a protocol which involved synchronisation of a culture of cdc7.4 by α-factor arrest, followed by release from the

α-block and splitting of the culture into two portions, both of which received 10 μCi/ml 6-^{3}H uracil. One portion was incubated for 90 minutes at 23°C and the other at 38°C. After the labelling period, an excess of unlabelled uracil was added to each culture, to chase any replicating intermediates. The sample preparation, as described by Livingston and Klein (1977), was a modification of the method of Hirt (1967), involving clearance of a crude lysate by precipitation at 0°C with SDS-M NaCl. At this point in the preparation, equal portions of a similarly processed culture of cdc7.4 which had been labelled overnight with 0.08 μCi/ml 2-^{14}C uracil were added to each sample to act as markers. The gradient analysis displayed in Fig. 38, shows conclusively that 2μm plasmid was not replicated in cdc7.4 cells held at the restrictive temperature. Likewise, nuclear DNA replication was blocked but mitochondrial DNA replication continued unabated, as had been shown previously (3.3.2.2). At 23°C, plasmid, nuclear and mitochondrial DNAs were replicated in proportion to the amounts of ^{14}C-prelabelled DNA.

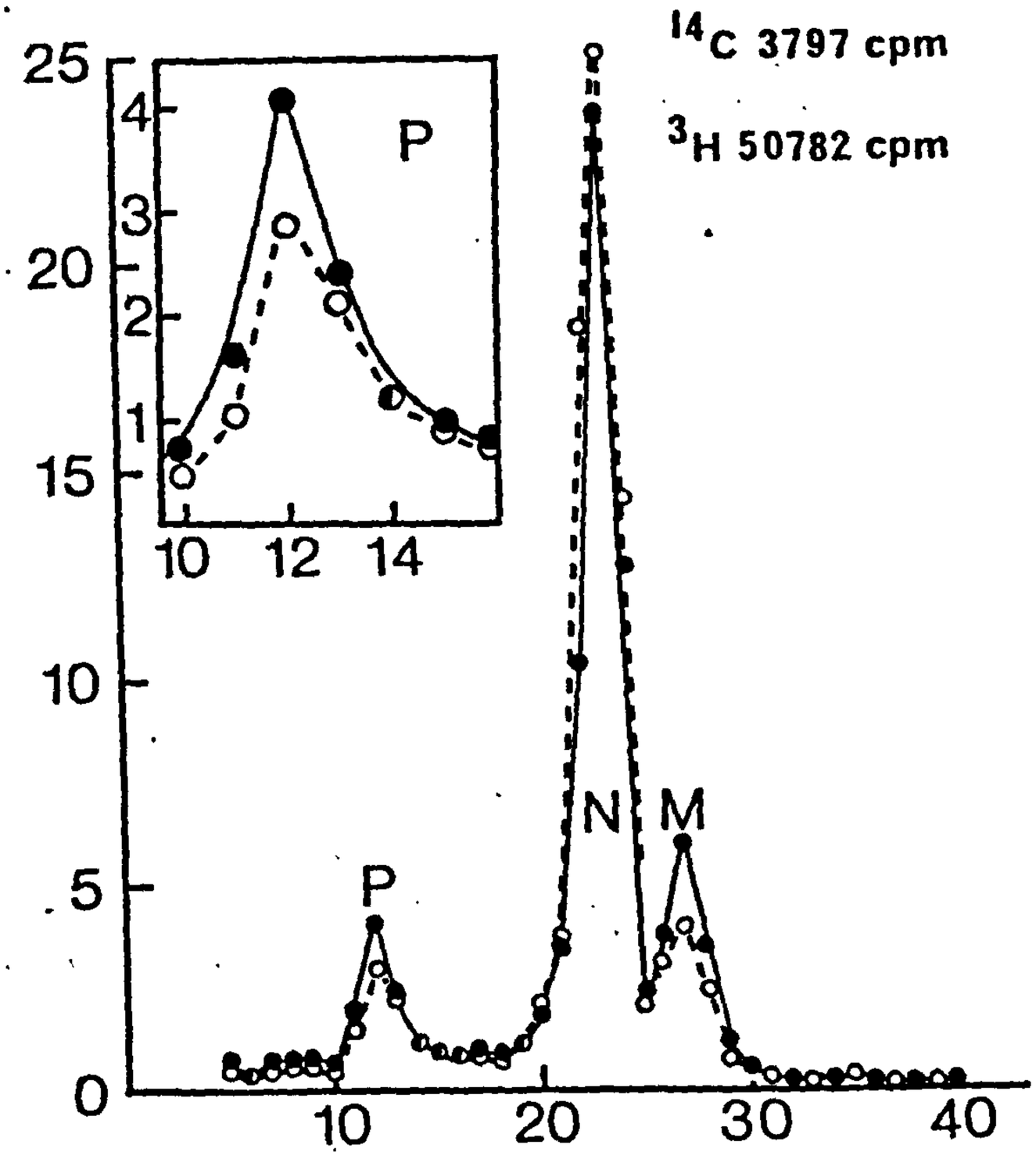
Confirmation that the peak designated "plasmid" (inset in Figs. 38, 39 and 41) contained 2μm plasmid, was obtained by electron microscopy and agarose gel electrophoresis. Molecules from a corresponding peak on another gradient were spread as a monolayer by the Kleinschmidt technique (2.18), then picked up on carbon coated E-M grids. Fig. 40 shows that the molecules observed were uniformly 2μm in length, mainly supercoiled, with an occasional relaxed circle, which probably arose during sample preparation for the microscope. This DNA migrates as a single band fractionally faster than DS581 ColE1 DNA on agarose gels, again confirming its size and nature (data not shown).

A modified protocol was used in similar experiments upon A364A, cdc4.3, cdc28.1 and cdc28.2. Cells from the overnight 2-^{14}C uracil

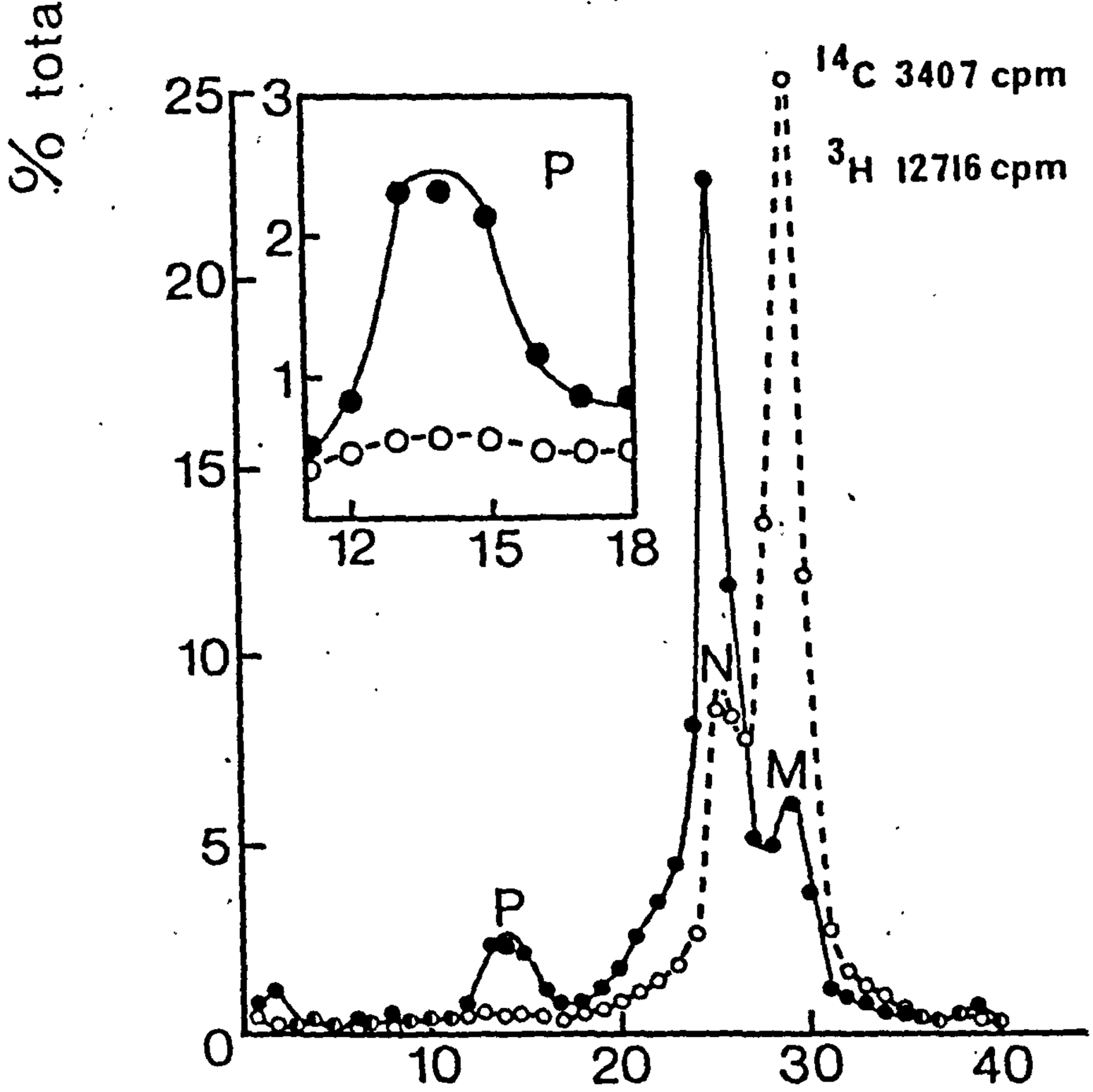
Figure 38: 2 μm plasmid replication in cdc7.4.

A 50ml exponential culture of cdc7.4 (DE200.1.3) at 10^7 cells/ml was arrested with α-factor then split and released at 23°C (a) and 38°C (b) in the presence of 10 μCi/ml 6-^{3}H uracil as described in Figure 13. After 90 minutes the label was chased for 30 minutes by the addition of 200mg/litre uracil to the cultures. Lysates were prepared as described in 2.15.2. To each lysate (2.5ml), 1.25ml of a similar lysate prepared from cdc7.4 grown for 5 generations in the presence of 0.08 μCi/ml 2-^{14}C uracil was added. Ethidium bromide-caesium chloride gradients were prepared and centrifuged as described in 2.15.2. The inset in each case shows the plasmid peak, P. N= nuclear DNA; M=mitochondrial DNA. (○) ^{3}H; (●) ^{14}C. Total ^{14}C and ^{3}H counts recovered on the gradients are given.

a. cdc 7·4 , 23°



b. cdc 7·4 , 38°



labelled cultures were washed free of radiolabel by centrifugation, then resuspended in fresh medium and used for the 6-^{3}H uracil labelling treatments. Also, since the cell cycle times of these strains are about 3 hours (data not shown), and the S phase lasts for about 25% of the cell cycle (Carter, 1975), S phase was estimated to be 45 - 50 minutes in exponential cultures growing at 23°C. Thus, the washed ^{14}C-prelabelled cultures were split and incubated at 23°C and 38°C (36°C for cdc4.3) for 65 minutes, calculated to allow all S phases underway to be completed at the restrictive temperature, prior to labelling with 6-^{3}H uracil for 2-5 hours. The results of this labelling regime upon the DNA synthesised at 38°C or 36°C in A364A, cdc4.3 and cdc28.2 are shown Figures 39 and 41. Companion gradients showing the DNA labelled at 23°C are not included here. The distribution always corresponded precisely with the ^{14}C-prelabelled DNA. Four points are apparent from these figures:

1. mitochondrial DNA was not always resolved from nuclear DNA;

2. DNA replication in A364A at 38°C followed the same distribution as the ^{14}C prelabel;

3. cdc4 showed a clear increase of mitochondrial DNA at 38°C, without a corresponding increase in nuclear and plasmid DNA.

4. there was not such a convincing shut-off of plasmid replication in cdc28.2 (Fig.39) as was seen in the other cdc mutants. However, the ^{3}H/^{14}C ratios for the peaks labelled p, n and m in Fig.39 are 0.67, 0.70 and 2.82 respectively. Clearly the plasmid was replicated to the same extent as the nuclear DNA at 38°C, both being much less than the mitochondrial DNA fraction. Control gradients containing DNA labelled in cdc28.2 at 23°C gave corresponding values of 3.87, 4.45 and 3.81 respectively. Thus 2μm plasmid DNA replication, like nuclear DNA replication, requires functional CDC28, CDC4 and CDC7 gene products for its initiation.

Figure 39: 2 μm plasmid replication in A364A and cdc28.2.

The experiment was the same as in Figure 38 except that the ^{3}H labelled cultures had themselves been pre-labelled with 2-^{14}C uracil overnight. The gradients show results for cultures labelled at 38°C, after 65 minutes at 38°C without label; (a) A364A, labelled for 70 minutes; (b) cdc28.2. labelled for 150 minutes. (○) ^{3}H; (●), ^{14}C. P, N and M = plasmid, nuclear and mitochondrial DNAs.

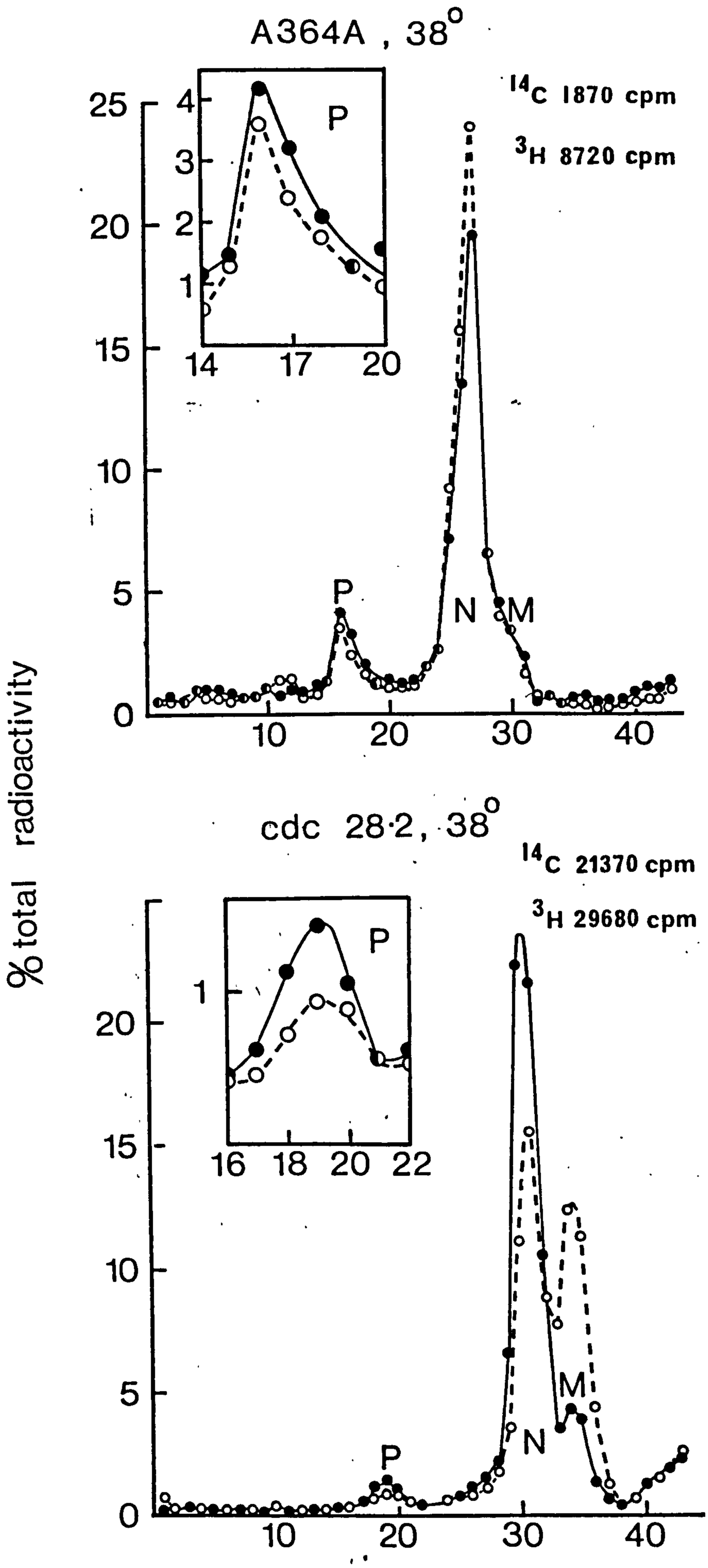

A364A , 38°
^{14}C 1870 cpm
^{3}H 8720 cpm
P
N
M
cdc 28·2, 38°
^{14}C 21370 cpm
^{3}H 29680 cpm
% total radioactivity
Fr. no.

Figure 40: Electron microscopy of the 2 μm plasmid peak on Etd Br-CsCl gradients.

The DNA present in the peak marked P. in Figure 38 was Kleinschmidt spread as in 2.18 except that staining was with phosphotungstic acid. A field of supercoils (a) was observed with occasional open circles (b) The bar represents 0.5 μm.

Figure 41: 2 μm plasmid replication in cdc4.1.

The protocol was the same as used in Figure 39, except that 6-^{3}H uracil labelling was for 2.5 hours at 36°C after 1 hour at 36°C without label. (O), ^{3}H; (●), ^{14}C. P, N and M = plasmid, mitochondrial and nuclear DNAs.

Figure 40

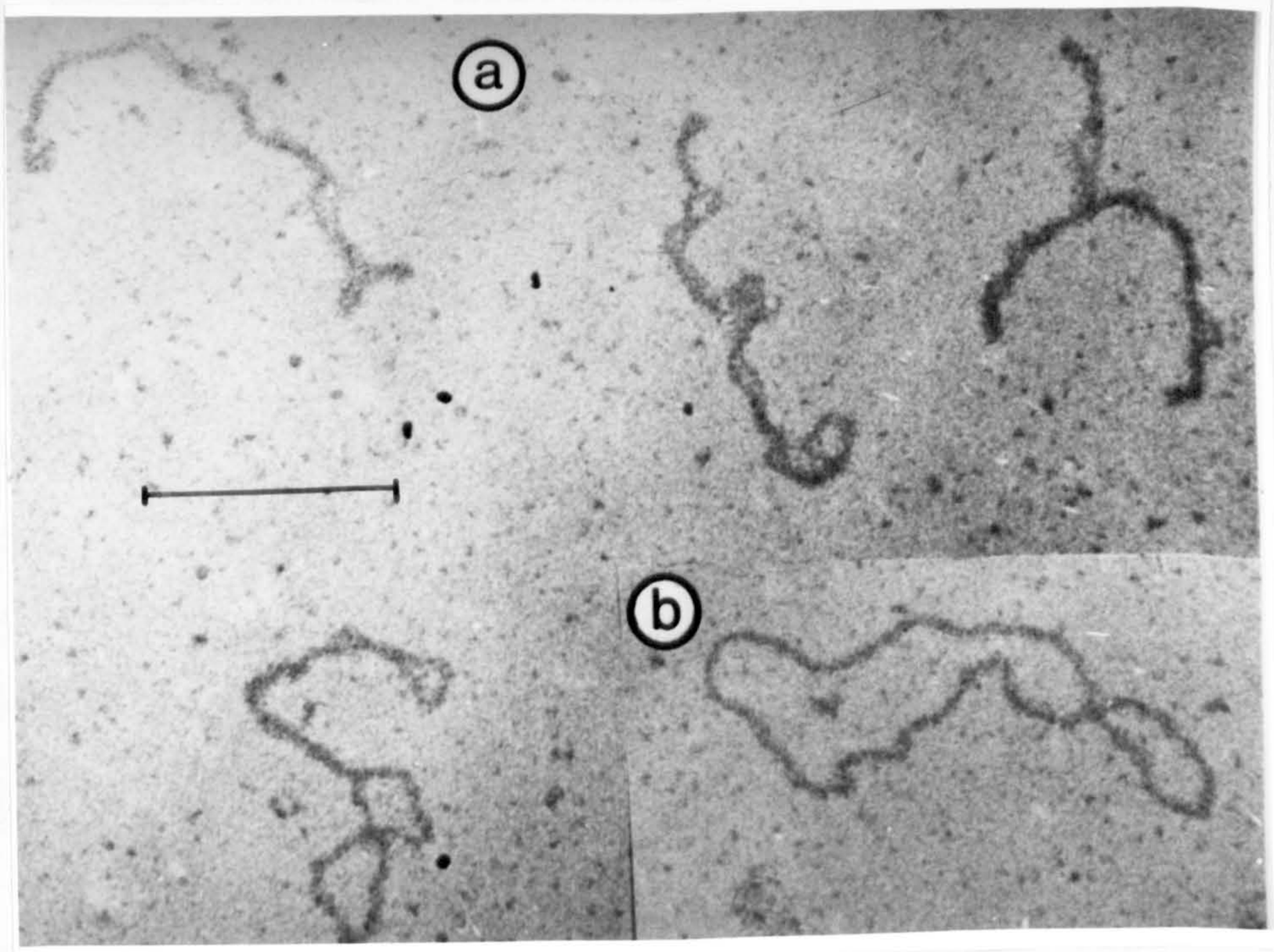
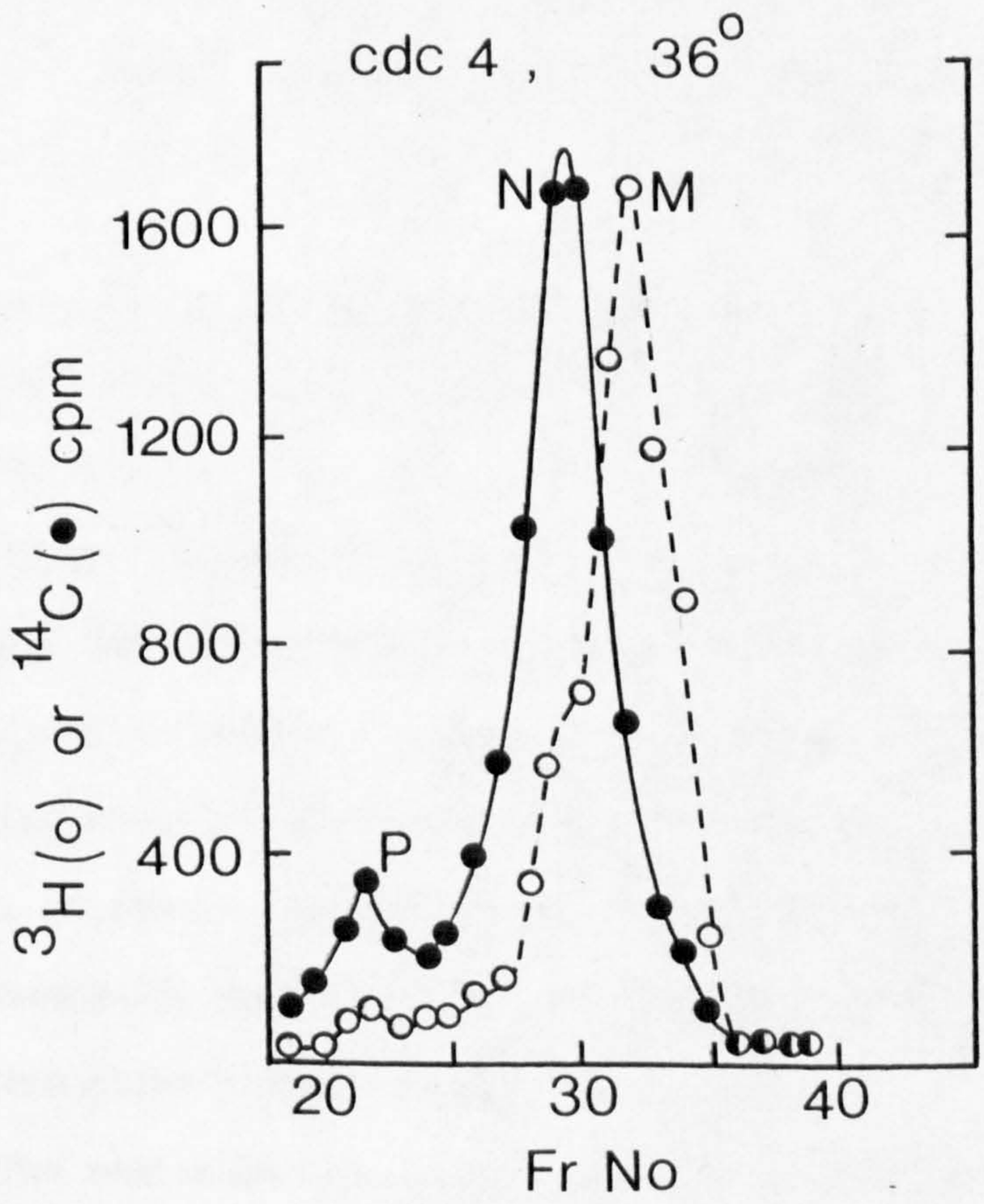


Figure 41



An interesting point which arose from these experiments was that the amount of 2 μm plasmid relative to total DNA varied between the strains examined. A364A, cdc4.1 and cdc7.4 all possessed roughly similar amounts (5-8% of the total DNA on each gradient), whereas cdc28.1 and cdc28.2 both contained reproducibly less plasmid (1-2% of total DNA per gradient). This probably reflects a lower copy number of the 2μm plasmid in these strains, which may be an effect of the cdc28 mutation. However, it must be noted that 2μm DNA sequences are totally absent in certain strains of S.cerevisiae, without apparent adverse effects (Tabak, 1977).

A difficulty encountered in these experiments was the sensitivity of cells grown at 38°C to Arthrobacter enzyme. Cultures grown at 23°C spheroplasted well, and could be handled without excessive lysis, but after growth at 38°C, cells of all the cdc mutant strains were very easily lysed. This did not affect the results presented here, but did cause invalidation of several other experiments. This lead also to the inclusion of 1.2M sorbitol in the sample preparation (2.15.2), which may be responsible for the occasional poor resolution of nuclear from mitochondrial DNA in caesium chloride.

5.3.4.2. Large scale preparation of yeast 2μm plasmid.

Attempts to scale up the ethidium-bromide caesium chloride separation method (2.15.2) for preparative purposes proved unsuccessful. One problem encountered when making 6-^{3}H uracil labelled plasmid was the high RNA contamination despite RNAase digestion before centrifugation. A solution to this was offered by the use of dTMP-permeable strains, allowing specific labelling of DNA (Jannsen et al, 1970; Brendel et al, 1975). A second improvement came with the development of chromatography on benzoylated-naphthoylated DEAE (BND) cellulose as an alternative plasmid separation technique.

The rationale behind the isolation of dTMP permeable mutants is

as follows. Depletion of tetrahydrofolates by treatment with aminopterin (causing inhibition of dihydrofolate reductase) and sulphanilamide (causing inhibition of de novo production of dihydrofolate) causes growth arrest. The biosynthetic pathways of adenine, histidine, methionine, and dTMP are tetrahydrofolate dependent (Barclay and Little, 1977). Administration of both inhibitors is necessary to arrest yeast growth (Brendel et al, 1975). If cells are starved for dTMP, continued growth in the presence of a metabolic block (i.e. with adenine and required amino acids supplied exogenously, and the aminopterin/sulphanilamide block imposed) results in "thymineless death" of the cells. Cells can be rescued if they are permeable to exogenously supplied dTMP, as a result of mutation at the tup locus (Wickner, 1974).

cdc21.1 (H146.2.3.) which was known to have a defective thymidylate synthetase (Bisson and Thorner, 1977), was exposed to the above selection conditions as described in 5.2.3. Large colonies were screened for their ability to incorporate ^{3}H-dTMP. One strain cdc21.1 DE46/2 gave optimum labelling, and the best conditions were 32°C in CAD medium containing 0.7 µg/ml dTMP and 0.25 µCi ^{3}H-dTMP/ml. Another similarly isolated strain (g308-6C) was obtained from Dr. J.G. Little, which proved to be even better at ^{3}H-dTMP incorporation, than cdc21.1 DE46/2. ^{3}H-dTMP was shown to have labelled DNA exclusively by caesium chloride gradient analysis.

The often poor yield of 2 µm plasmid from EBr-CsCl gradients, caused by the overlap of the peaks as the gradient load was increased, prompted a search for alternative plasmid separation techniques. Both the hydroxyapatite column method of Colman et al, (1978) and the acid phenol extraction technique of Zasloff et al, (1978) proved unsuccessful when applied to yeast. Another method involved the use of benzoylated, naphthoylated DEAE (BND) cellulose which binds double-stranded DNA much less tightly than single stranded DNA. Using alkali to denature

linear DNA, followed by renaturation and chromatography on BND-cellulose, in Sinsheimer and Komano (1968) separated ØX174 RF1 DNA from the linear form. Hayton et al (1973) had also used the method to detect single stranded regions in replicating DNA in BHK cells.

The preparation is described in detail in 5.2.4. Briefly it consisted of : (1) spheroplast formation using Arthrobacter enzyme (2.9.3.) and lysis by 1% sarkosyl detergent; (2) high speed centrifugation to yield a cleared lysate; (3) precipitation of nucleic acids by 10% PEG 6000 in MNaCl; (4) RNAaseA and proteinase K digestion of the resuspended PEG precipitate; (5) denaturation at pH 11.85 and room temperature for 3 minutes followed by neutralisation and rapid chilling; (6) BND-cellulose chromatography, eluting plasmid DNA with 1M NaCl in 1mM Tris-HCl pH 8.0, 1mMEDTA, and denatured DNA with 6M guanidinium chloride.

A typical purification is shown in Table X. The final yield represents about 20% of the total plasmid in the cells. Samples taken at various stages during the purification were run on a 1% agarose gel (Fig 42). The 1M-NaCl wash contained only supercoiled plasmid, but some was also retained on the column, and was eluted with 6M guanidinium chloride. Extensive washing of the column before elution was necessary to remove RNA, which does not appear to stick. 2 μm plasmid prepared in this manner was used in the assays for endonuclease activity 5.3.5.

An interesting point arising from the use of dTMP permeable strains was that the specific activity of the labelled DNA was never more than 15% of that of the input radioactivity in the growth medium. This suggests that there is endogenous thymidylate synthetase activity in both g308-6C and cdc21.1 DE46/2. Bisson and Thorner (1977) assayed thymidylate synthetase activity in cdc21 extracts, and found less than 0.2% of the activity of comparable wild-type extracts, even at 23°C. F.Z. Watts has confirmed this observation. The evidence cited

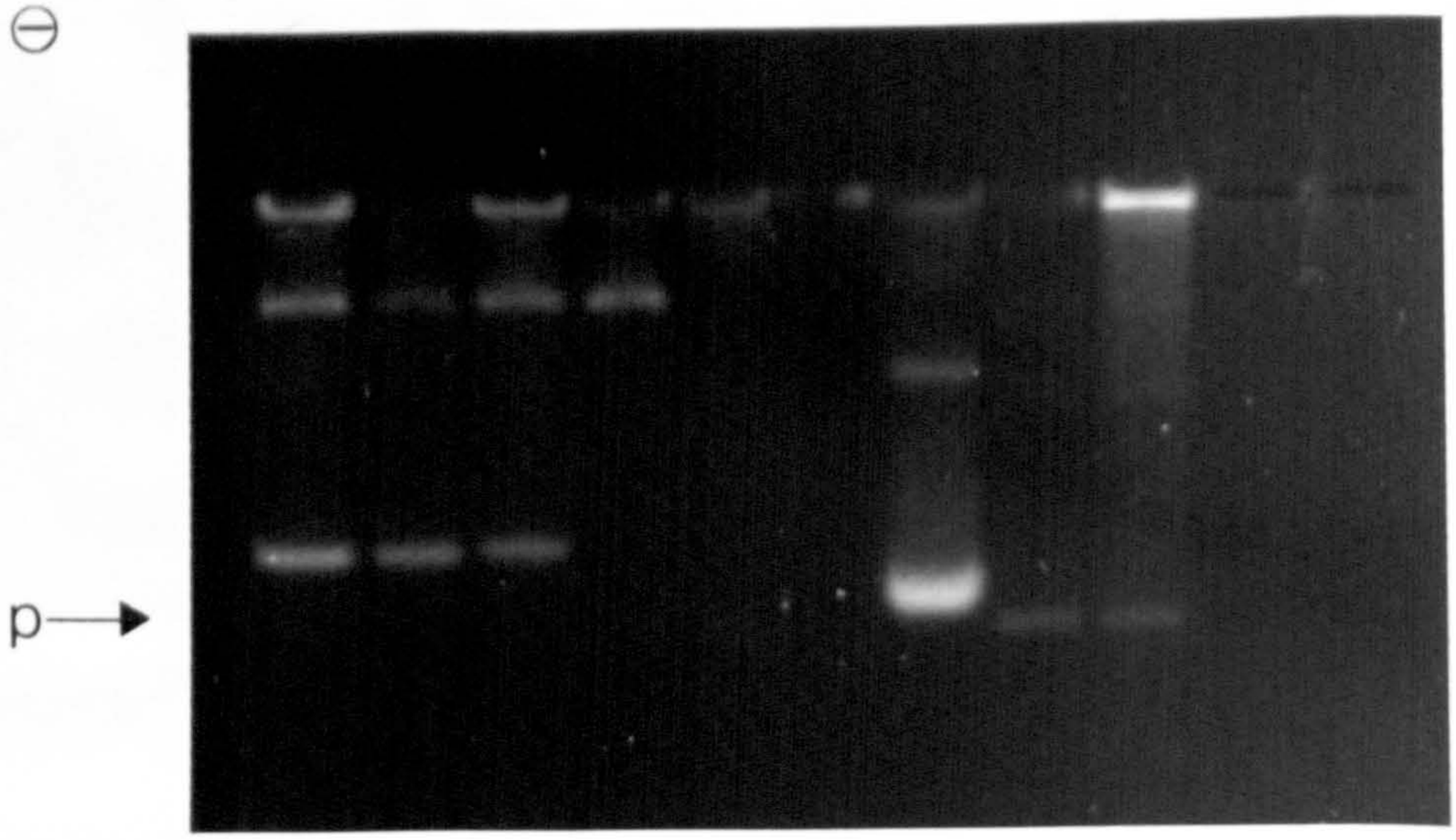


Figure 42: Agarose gel electrophoresis of samples from BND-cellulose purification of the 2 μm plasmid.

A 1% agarose gel of samples taken during the purification procedure reported in Table X was run as described in 2.17. Sample volumes were 10-25 μl. Tracks 1-5 correspond to samples 3,4,5,6 and 8 in Table X; track 6 was the flow through from BND-cellulose; track 7 was 2 μg ColE1 DNA (4.2×10^6 MW) as a marker; tracks 8 and 9 were the 1M NaCl and 6M guanidinium chloride washes from BND-cellulose. p, 2 μm plasmid.

here, coupled with the fact that cdc21 survives at the permissive temperature, demonstrates the presence <u>in vivo</u> of a process supplying dTMP. The apparent absence of activity <u>in vitro</u> must be caused by instability of the mutant enzymes during extraction. This must be borne in mind when attempting to isolate mutant proteins.

Table X. BND-cellulose purification of 2 μm plasmid.

Sample	Description	Total TCA precipitable ^{3}Hcpm ($x10^{-3}$)	Recovery as % of total ^{3}Hcpm in culture
1.	Culture	15,084	100
2.	Digest supernatant	79	0.5
3.	Lysate (pre Spinco)	3,570	23.7
4.	Lysate (post Spinco)	3,220	21.3
5.	Spinco pellet	4,160	27.6
6.	PEG precipitate	3,144	20.8
7.	PEG supernatant	39	0.3
8.	Post-alkali treatment (BND cellulose load)	2,492	16.5
9.	M-NaCl wash	98	0.6
10.	6M guanidinium chloride wash	1,451	9.6

Notes. A. Sample 3 was low, presumably due to lack of scintillant penetration on the filters, since it should equal the total of 4 and 5.

B. The strain used was g308-6C, labelled with (methyl-^{3}H)-thymidine 5' monophosphate. Specific activity of DNA in 9 and 10 was 2720 cpm/μg.

5.3.6. Endonuclease activity.

For the purpose of screening a large number of gradient fractions, the endonuclease assay used was the nitrocellulose filter binding assay described by Center et al (1970). This measures activity on supercoiled covalently closed circular DNA by its conversion to a form which can be retained on nitrocellulose filters. These filters preferentially trap single-stranded DNA at high salt concentrations (6xSSC). The assay was used by Henry and Knippers (1974) in the purification of ØX174 gene A activity. Assays were short (3 minutes), since the hypothetical endonuclease need only nick once per circle. Since the initiation of DNA synthesis in vivo proceeds rapidly upon a temperature shift from 38°C to 23°C (3.3.2.1), it was necessary that the reaction mixtures and fractions under test both be preincubated at thereaction temperatures, to ensure that the defective protein could not function.

5.3.6.1. Fractionation of crude extracts on DEAE-cellulose.

Crude extracts (5.2.7) of log-phase cdc7.4 cells were fractionated by ion-exchange chromatography on DEAE-cellulose DE52, and assayed using yeast ^{3}H-2 μm plasmid as substrate. The first experiment showed some apparent temperature-sensitivity of an early eluting peak (50mM NaCl, fractions 32-38) whereas late eluting activity was remarkably similar at both 23°C and 38°C (Fig 43). However, when fractions 33-36 were pooled and chromatographed on phosphocellulose, a single peak was eluted at 0.4M NaCl which showed no apparent temperature sensitivity in the filter binding assay (data not shown).

On repetition of the DEAE-cellulose fractionation of a cdc7.4 crude extract, assaying for endonuclease activity with BND-cellulose prepared ^{3}H-2 μm plasmid as substrate, (Fig 44), another marginally ts peak eluting at the front of the gradient was seen. However, on careful reassay of fractions 67-74 covering this region, the effect could not be reproduced. It was thought that a contributory factor

Figure 43: Nitrocellulose filter binding assay for endonuclease activity in fractions from DEAE cellulose using 2 μm plasmid DNA as substrate.

A crude extract (18ml) from 8.4g wet weight of log-phase yeast cells prepared as described in 5.2.7, was applied onto a 3.0x29.5 cm DEAE-cellulose column at a rate of 40ml/hour. After extensive washing with TEDGP Buffer, pH8.0 (2.12), bound proteins were eluted with a 200ml 0-1M KCl gradient in the same buffer. 50 μl samples from the fractions were assayed by endonuclease activity at 23°C (O) and 38°C (●) using the nitrocellulose filter binding assay described in 5.2.6 with ^{3}H-2 μm plasmid DNA (11800cpm/ μg; 970cpm/assay) as substrate.

Figure 44: Nitrocellulose filter binding assay for endonuclease activity using 2 μm plasmid DNA as substrate.

The experiment was the same as Figure 43, except that the crude extract volume was 42ml, a 2.8x36cm DEAE-cellulose column was used and the ^{3}H-2 μm plasmid was 1680cpm/ μg (860cpm/assay) (O), 23°C; (●) 38°C.

Figure 43

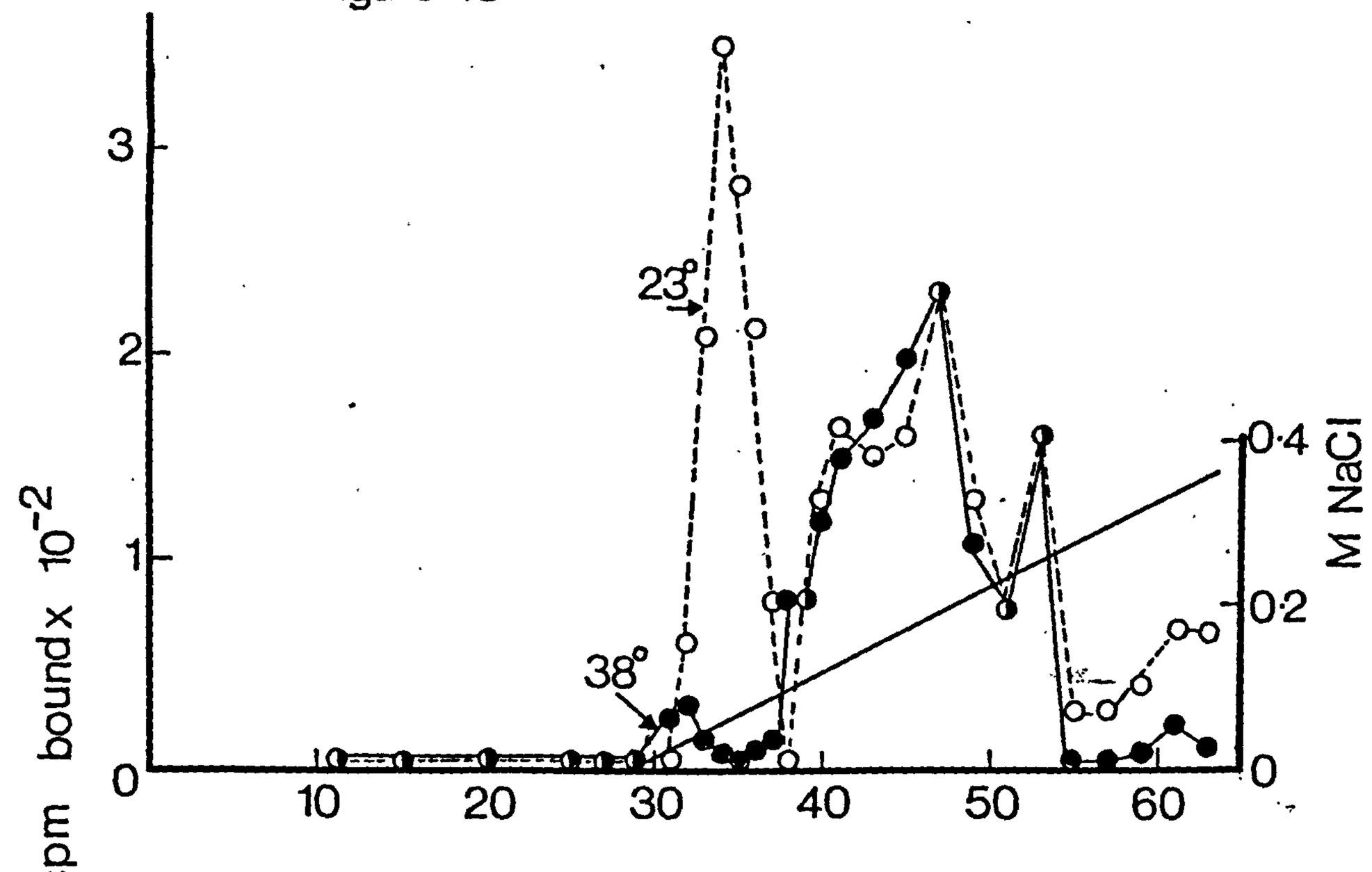
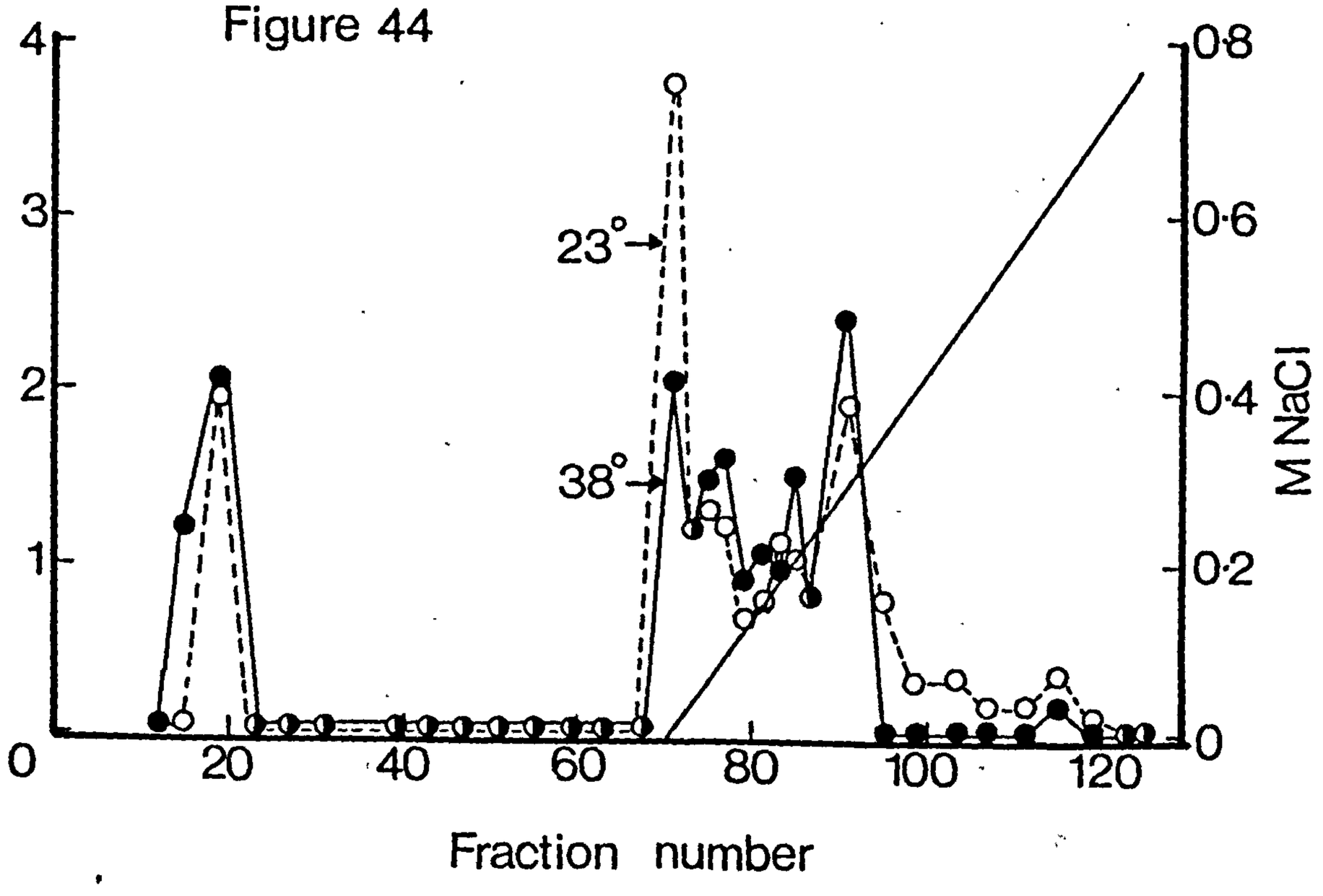


Figure 44



in this lack of reproducibility was the high protein concentration used in these assays, which upon denaturation may have trapped DNA on the filters. This was particularly so in the mid-range of the gradient, where assays contained up to 0.5mg of protein.

When assays were repeated after ten-fold dilution of the fractions using enzyme diluent (20mM Tris-HCl pH8.0, 0.5mM EDTA, 0.5mg/ml BSA heated at 75°C for 45 minutes, 1mM DTT), and performed on ^{3}H-DS581 ColE1 DNA, a much clearer pattern emerged (Fig 45). With the exception of fractions 73 and 75, all other fractions assayed had greater activity in the filter binding assay at 38°C than at 23°C. A small peak at fraction 81 at 23°C was seen, and a much larger peak, corresponding to 70% retention of the input DNA, was seen for fraction 85 at 38°C. These assays, at the same dilutions, were repeated using ^{3}H-pJDB219 DNA as substrate (Fig 46). Minor peaks at fractions 19 and 71 appeared which were not seen using ColE1 DNA, otherwise the activity profiles of Figs 45 and 46 were similar. Identical assays to those of Fig 45, i.e. using ColE1 DNA, were performed, but instead of boiling and chilling as in the endonuclease assay (5.2.7), chilled 6xSSC was added directly and the samples were filtered through nitrocellulose (Fig 47). In such high salt conditions, and without denaturation, the assay measured tight DNA binding proteins. The major peak for fraction 85 at 38°C is a feature of Figures 45, 46 and 47. It had been assumed throughout these assays that the retention of counts on the filters represented endonuclease activity. The unexpected amount of tight DNA binding activity made this assumption questionable.

The products of the reactions were therefore analysed on agarose gels (2.17), to ascertain that the supercoiled plasmid substrate was being nicked by endonuclease action. Also, to establish whether the differences between Figs 44, 45 and 46 were due to artefacts in the assay, or were due to DNA sequence specific effects, assays were

Figure 45: Nitrocellulose filter binding assay for endonuclease activity using ^{3}H-ColE1 DNA as substrate.

The fractions from the DEAE-cellulose gradient in Figure 44 were diluted 10 fold with 10mM Tris-HCl, pH7.6; 1mM EDTA; 100µg/ml BSA (heated at 75°C for 45 minutes); 1mM DTT; and assayed for endonuclease activity at 23°C (O) and 38°C (●) with ^{3}H-ColE1 DNA (8200cpm/ µg; 1680cpm/assay) as substrate.

Figure 46: Nitrocellulose filter binding assay for endonuclease activity using ^{3}H pJDB219 DNA as substrate.

The experiment was the same as Figure 45 except that ^{3}H-pJDB219 DNA (9200cpm/ µg; 1250cpm/assay) was used.

Figure 45

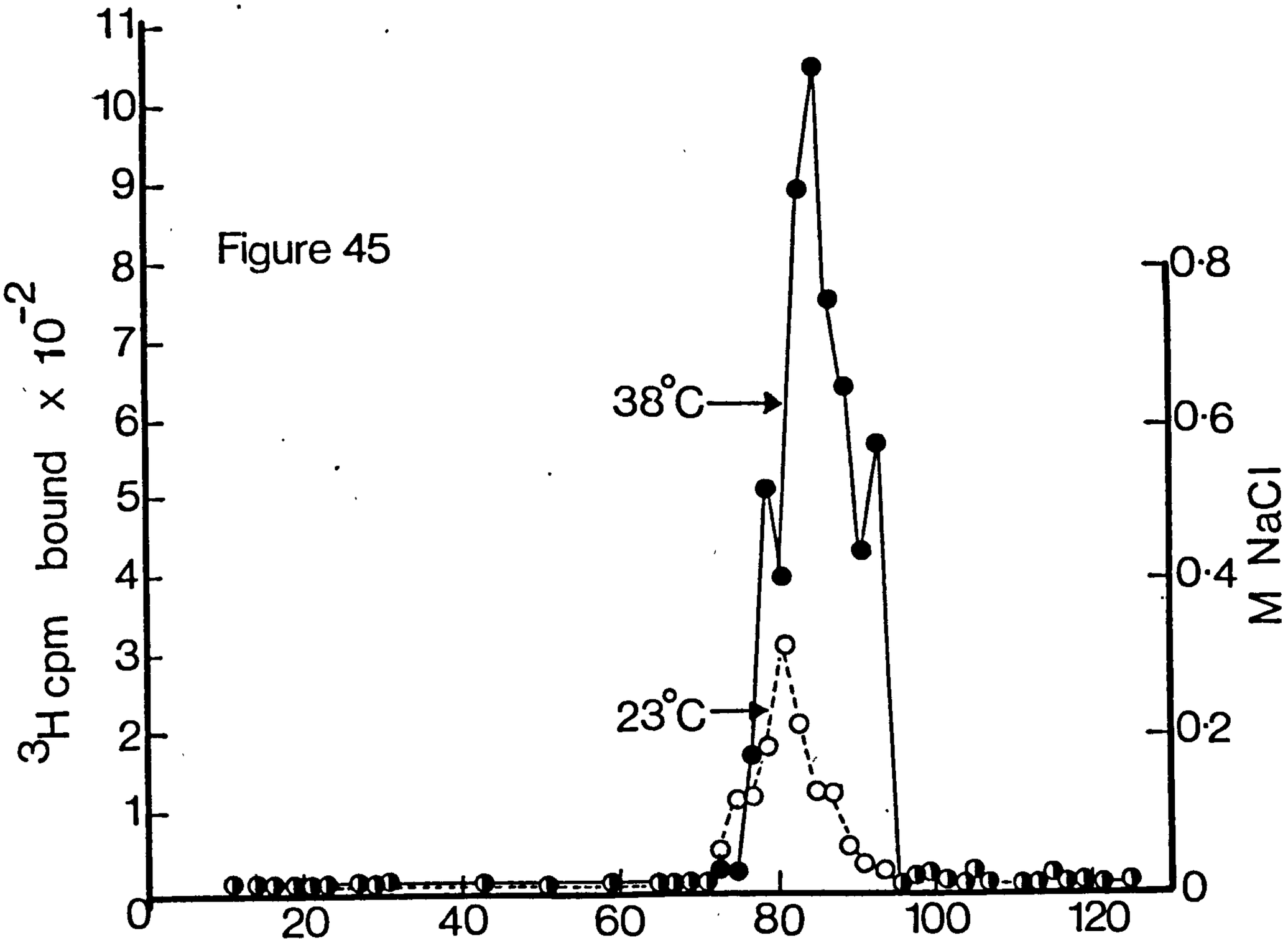


Figure 46

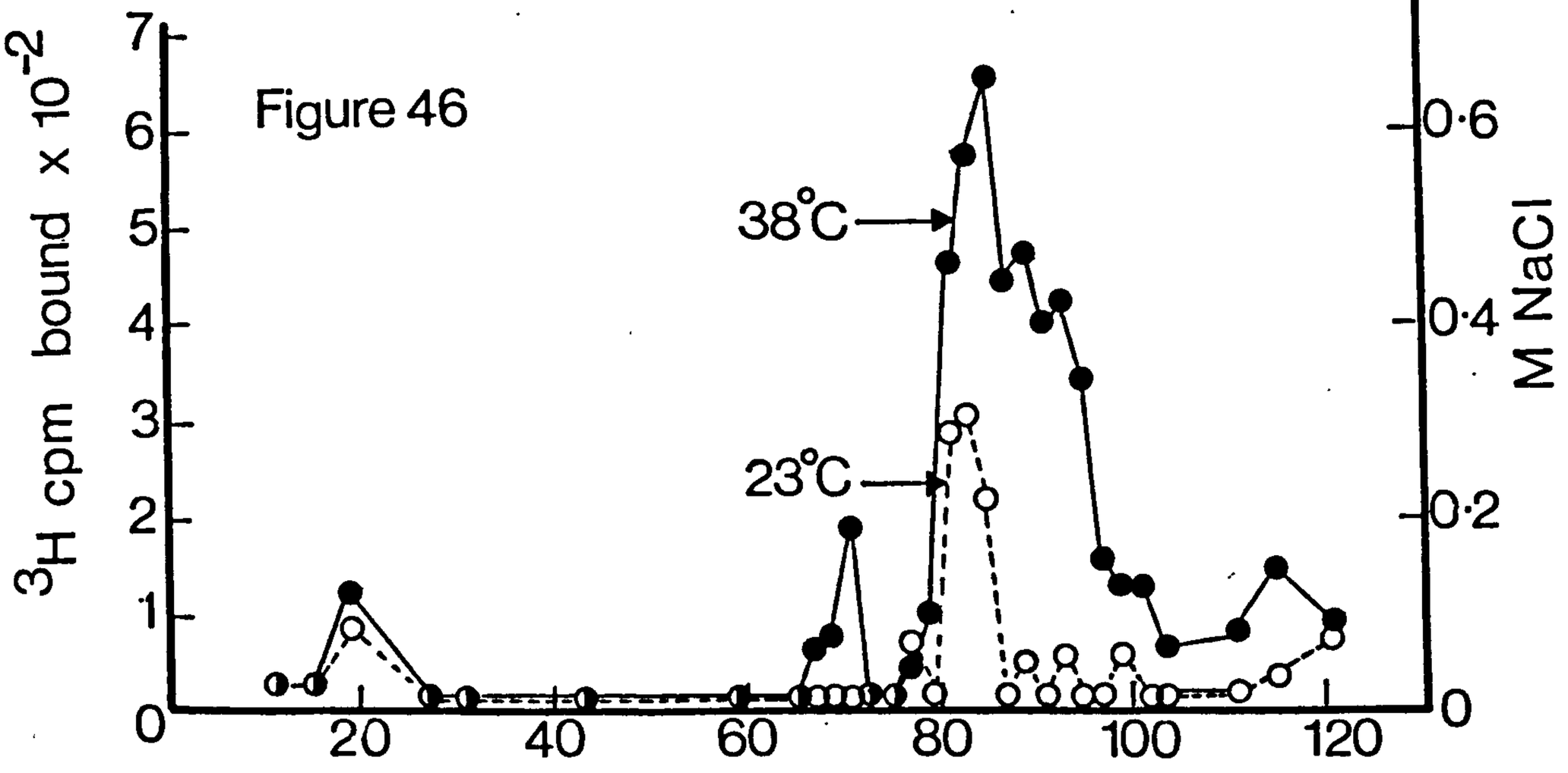


Figure 47: Nitrocellulose filter binding assay for double-stranded DNA binding proteins using ^{3}H-ColE1 DNA.

Reactions were performed using the same fractions and under the same conditions as for Figure 45, except that samples were not denatured by boiling prior to addition of 5ml 6xSSC and filtration through nitrocellulose.

Figure 48: Agarose gel electrophoresis of endonuclease assay products using ColE1 and pJDB219 DNA as substrates.

Fractions 67-85 from the DEAE-cellulose gradient in Figure 44 were diluted three fold with enzyme diluent as used in Figure 45, and 10 µl aliquots were incubated in 20 µl assays containing 50mM Tris-HCl, pH8.0; 7mM $MgCl_2$; 1.5mM DTT and 80 µg/ml pJDB219 DNA or 80 µg/ml ColE1 DNA. Incubations were performed at 38°C for 3 minutes. Reactions were terminated and samples electrophoresed in a 1% agarose gel as described in 2.17, except that 0.5 µg/ml ethidium bromide was included in the gel and electrophoresis buffers. Assays using fraction 81 contained 0.75mg/ml protein. P = pJDB219, C = ColE1. The band order in decreasing mobility was supercoil, linear, and opencircular DNAs.

Figure 47

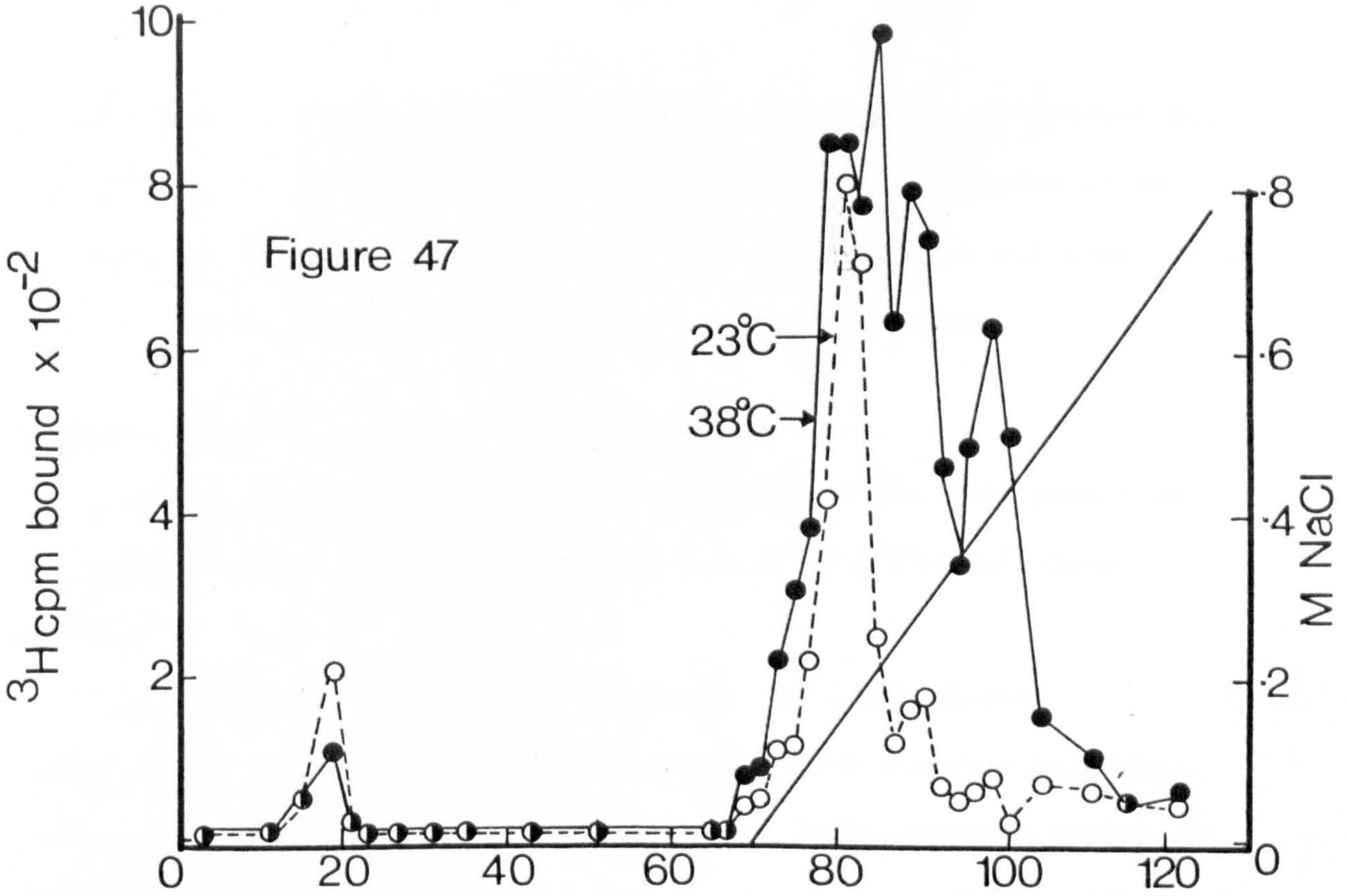
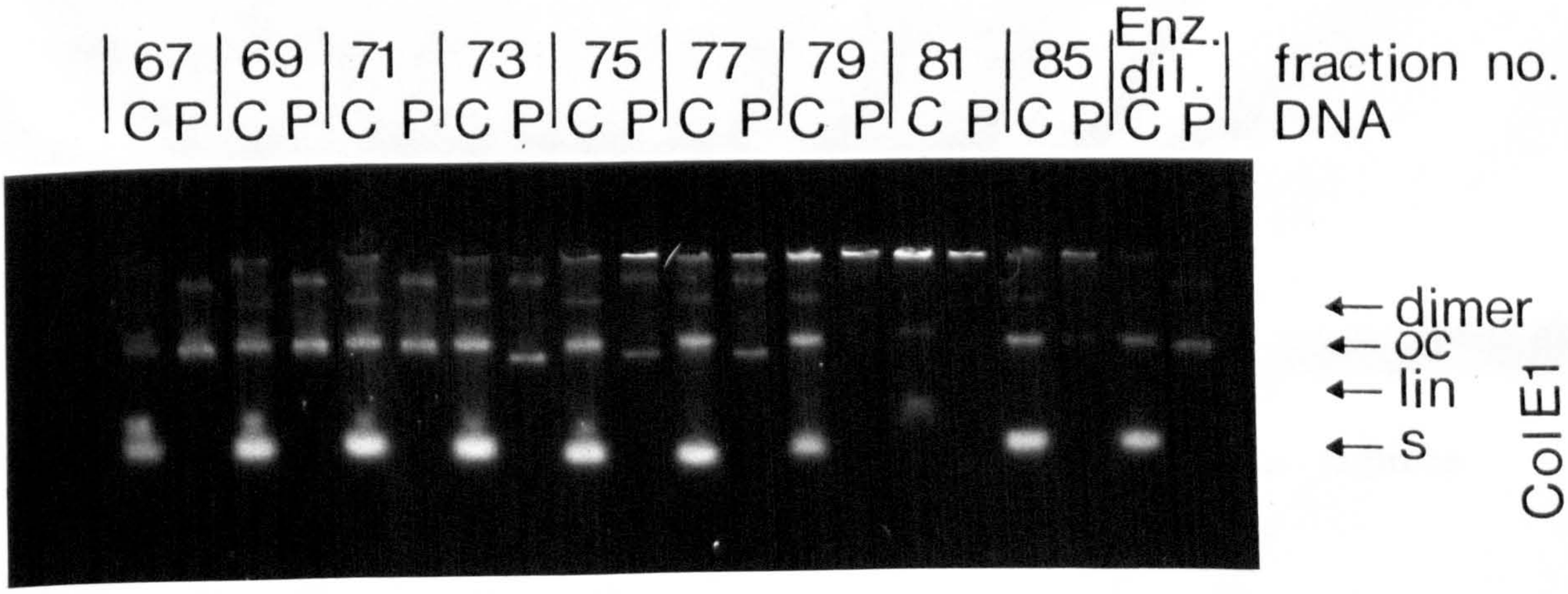


Figure 48



performed on both pJDB219 and ColE1 DNAs. The results of agarose gel electrophoresis of reactions using fractions 67-85 performed at 38°C is shown in Fig 48. The designations supercoil, linear and open circle were confirmed independently by DNAseI digestion patterns.

5.3.6.2. Survey of data from Figures 44-48.

Several points can be made from analysis of the data presented in Figures 44-48, which all derive from the same DEAE-cellulose gradient.

1) From Figure 48, both the pJDB219 and ColE1 plasmid preparations were partially nicked yielding open circular molecules, either before or during the reactions. The filter binding assays of Figure 46 using pJDB219 as substrate had fairly high blanks (250-300cpm from 1700cpm input DNA per assay), probably reflecting the amount of open circular DNA present in the preparation. Experience showed that ^{3}H-plasmid DNA was much more easily nicked on storage than unlabelled DNA.

2) The small filter binding effect of fraction 71 seen in Figure 46 which was not observed using ColE1 DNA as substrate (Figure 45) could not be observed on the agarose gel in Figure 48.

3) Fractions 73, 75 and 77 caused faster migration of the supercoiled band on the agarose gel (Figure 48).

4) Fraction 81 caused trapping of DNA in the sample well in Figure 48.

5) The retention of radioactive DNA on filters seen in Figures 45 and 46 using fraction 85 (the peak tube) at 38°C was not corroborated by the appearance of greater amounts of open circular DNA on the agarose gel in Figure 48. Thus it seems likely that some of the radiolabel retention caused by fraction 85 in Figures 45 and 46 was caused by the tight binding effect seen in Figure 47, and was not due to endonuclease activity. Bryant and Haynes (1978) reported a similar problem when using the Center et al (1970) nitrocellulose-

filter technique for assays of endonuclease activity in crude fractions.

5.3.6.3. Further studies on fractions 75 and 81.

The effects of fractions 75 and 81 described above demanded further analysis. Initially, it was thought that the fraction 81 effect, causing trapping of DNA in the sample well in Figure 48, was due to complex formation between the plasmid and saturating amounts of double-strand specific DNA binding protein, as was later shown for a Drosophila satellite DNA binding protein derived from Drosophila melanogaster embryos (Hsieh and Brutlag, 1979). However, it was discovered that the effect was due to precipitation in the reaction mix. The precipitation was due to the addition of 7mM $MgCl_2$ to fraction 81, and was independent of added DNA (data not shown).

When smaller amounts of fraction 81 were used (4.5 μg protein/ assay) in endonuclease assay reaction mixtures, the precipitation and consequent trapping of DNA in the sample wells of agarose gels was reduced. Fig 49 shows a time course of the effect of diluted fraction 81 upon pJDB219 at 38°C. Also shown is the effect of DNAase I upon the same plasmid (tracks 10 and 11). Clearly fraction 81 possessed endonuclease activity which was not thermosensitive.

The leading fractions of the same gradient (Figure 44) were assayed for DNA polymerase activity, using the assay described in 2.14. The activity peak, eluting at 0.12MNaCl was found to be at fraction 82 (Figure 50).

The purification of nicking-closing enzyme (topoisomerase) from S.cerevisiae was reported by Durnford and Champoux (1978). Using DEAE-Sephadex gradient sievorptive chromatography, the nicking-closing activity, as determined by a filter binding assay (Champoux and Durnford, 1975) eluted at the front of the salt gradient. The activity discovered here which caused faster migration of plasmid DNA on agarose gels (fraction 75; Figure 48) was eluted from DEAE-cellulose

Figure 49: Demonstration of endonuclease activity in fraction 81 by agarose gel electrophoresis.

A single 240 µl incubation was performed at 38°C of the same composition as used in Figure 48 except that the level of pJDB219 DNA was reduced to 64 µg/ml, and the amount of fraction 81 was reduced to 150 µg/ml of protein. At zero time the diluted fraction 81 was added and 30 µl samples were removed into 20 µl stop mix (2.17) at 0, 3, 5, 10, 15, 20, 25 and 30 minutes into the reaction (tracks 2-9). Tracks 9 and 10 were samples removed from a parallel digest containing 6ng/ml DNAase I (Sigma). Track 1 contained 0.8 µg linear pJDB219 DNA prepared by Sma I digestion (Boehringer). S = supercoil, l = linear, oc = opencircle. Electrophoresis was as described in Figure 48.

Figure 50: DNA polymerase activity.

The leading fractions of the DEAE-cellulose gradient shown in Figure 44 were assayed for DNA polymerase activity at 30°C using the assay described in 2.14.

Figure 49

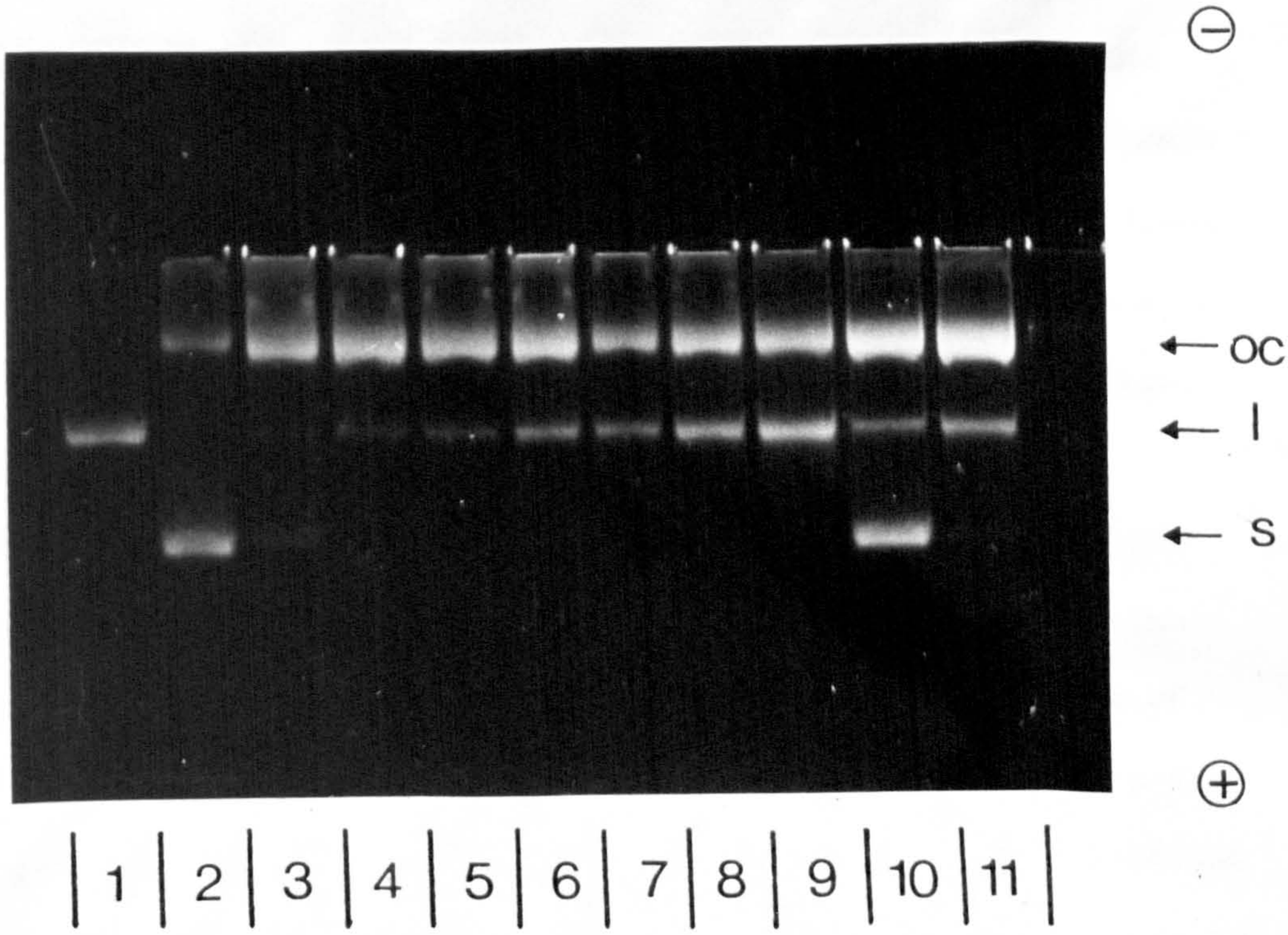
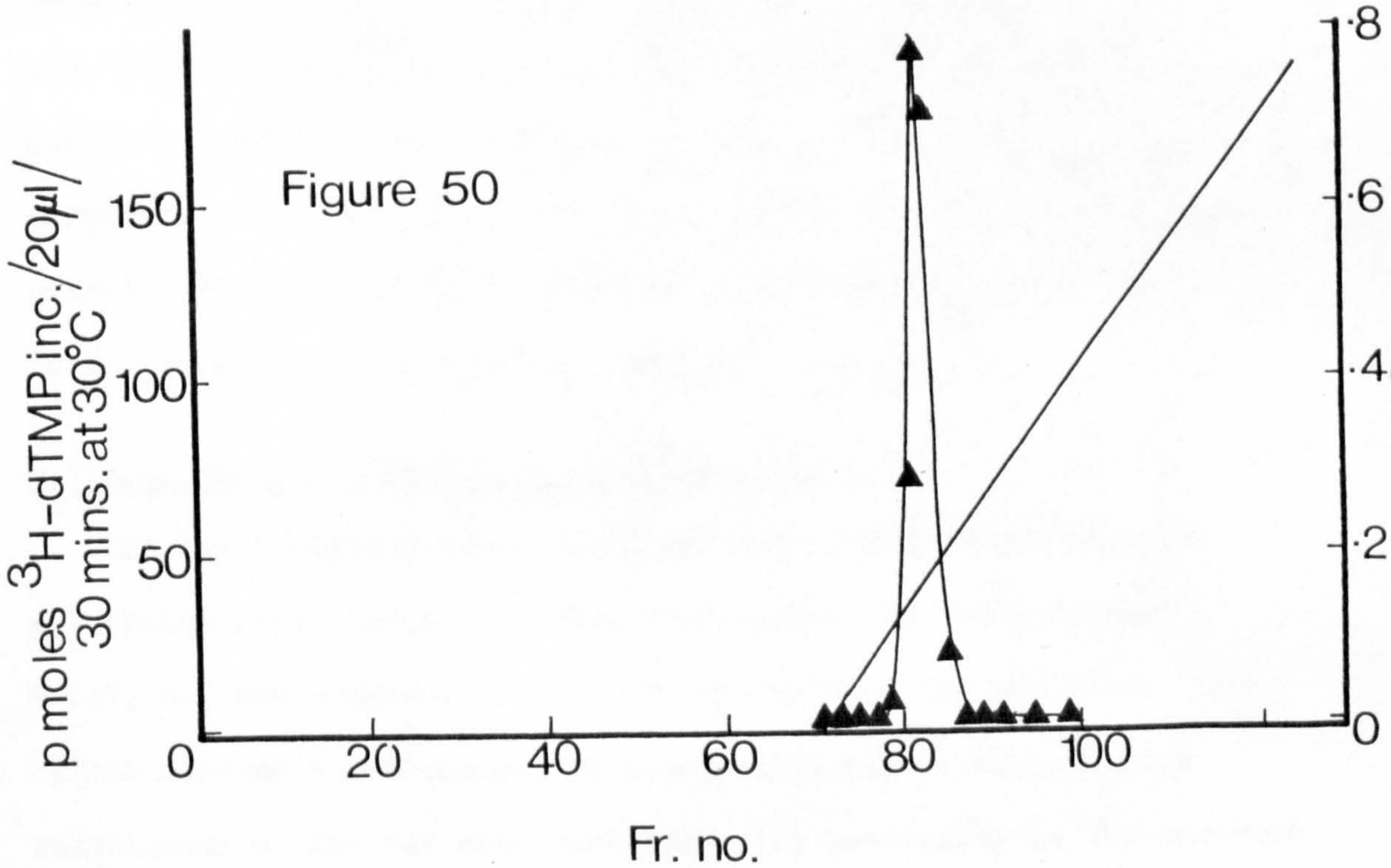


Figure 50



by low salt concentrations (50mM NaCl). It was thought that the activities might correspond. Consequently, the Durnford and Champoux (1978) filter binding assay was repeated over the leading fractions of the DEAE-cellulose gradient shown in Figure 44. However, their assay did not allow identification of fraction 75 as the nicking-closing enzyme.

The apparent ability of fraction 75 to cause supercoiled DNA to migrate faster on agarose gels presented a major problem. The effect was reproducible, but did not occur in the reaction mix of Durnford and Champoux (1978), which further suggested that this was not the nicking-closing enzyme (data not shown). If, after reaction at 38°C, reaction mixtures were exposed to protein denaturing conditions (5M guanidium chloride, 2% SDS, phenol extraction), the DNA retained its fast migrating characteristic (Figure 51). Thus this effect was due to a topological alteration of the plasmid and was not due to DNA/protein complex formation. The effect was shown to be not thermosensitive, by the appearance of the fast migrating species at both 23°C and 38°C (Figure 52). Other work showed that this activity was not affected by the addition of nalidixic acid at 300μg/ml or novobiocin at 80 μg/ml (Figure 51) or 1mMATP or 16mMEDTA (data not shown). Thus the 7mM Mg^{2+} present in the assay was not in fact required. This activity will be discussed again in 6.3.4.6.

5.3.6.4. Summary of Endonuclease assay work.

In the foregoing work, two techniques were used to estimate endonucleolytic cleavage of DNA: a nitrocellulose filter binding assay, and the analysis of reaction products on agarose gels. The filter binding technique was adversely affected by high protein concentration, and may also have responded spuriously in the presence of tight DNA binding proteins. Agarose gel separation provided a less ambiguous analysis, but could not be easily quantitated. Sucrose

Figure 51: Effect of protein denaturing conditions on the DNA product after reaction with fraction 75.

Eight 30 µl incubations were performed at 38°C for 3 minutes as described in Figure 48, except that pJDB219 was used at 106 µg/ml. Four received 9 µg fraction 75 protein from the DEAE-cellulose gradient in Figure 44, and the other four received enzyme diluent (Figure 45) as controls. Pairs of samples (control and fraction 75 treated respectively) were given the following treatments: 5M guanidinium chloride (tracks 1 and 2); 2% SDS and heating to 70°C for 2 minutes (tracks 3 and 4); heating to 70°C for 2 minutes (tracks 5 and 6); and extraction with phenol and chloroform (tracks 7 and 8). The samples in tracks 1-4 were also extracted with phenol and chloroform prior to electrophoresis as in Figure 48.

The same reaction mix was used for the samples in tracks 9-11, which all received 9 µg fraction 75. Track 9 was without further additions, track 10 contained 300 µg/ml nalidixic acid (Sigma) and track 11 contained 80 µg/ml novobiocin. After 3 minutes at 38°C these samples were treated as in Figure 48. S = supercoil, oc = open circle.

Figure 52: Temperature sensitivity of the fraction 75 effect.

30 µl assays were performed as in Figure 51 using 10 µl of three fold diluted fractions 73-77 from the DEAE-cellulose gradient in Figure 44. This was 12 µg protein for fraction 75. Pairs of reactions at 23°C and 38°C were performed for 3 minutes, then terminated and electrophoresed as in Figure 48.

The fractions were 18 months old when these reactions were performed and fraction 75 had suffered repeated cycles of freezing and thawing with consequent reduction in activity.

Figure 51

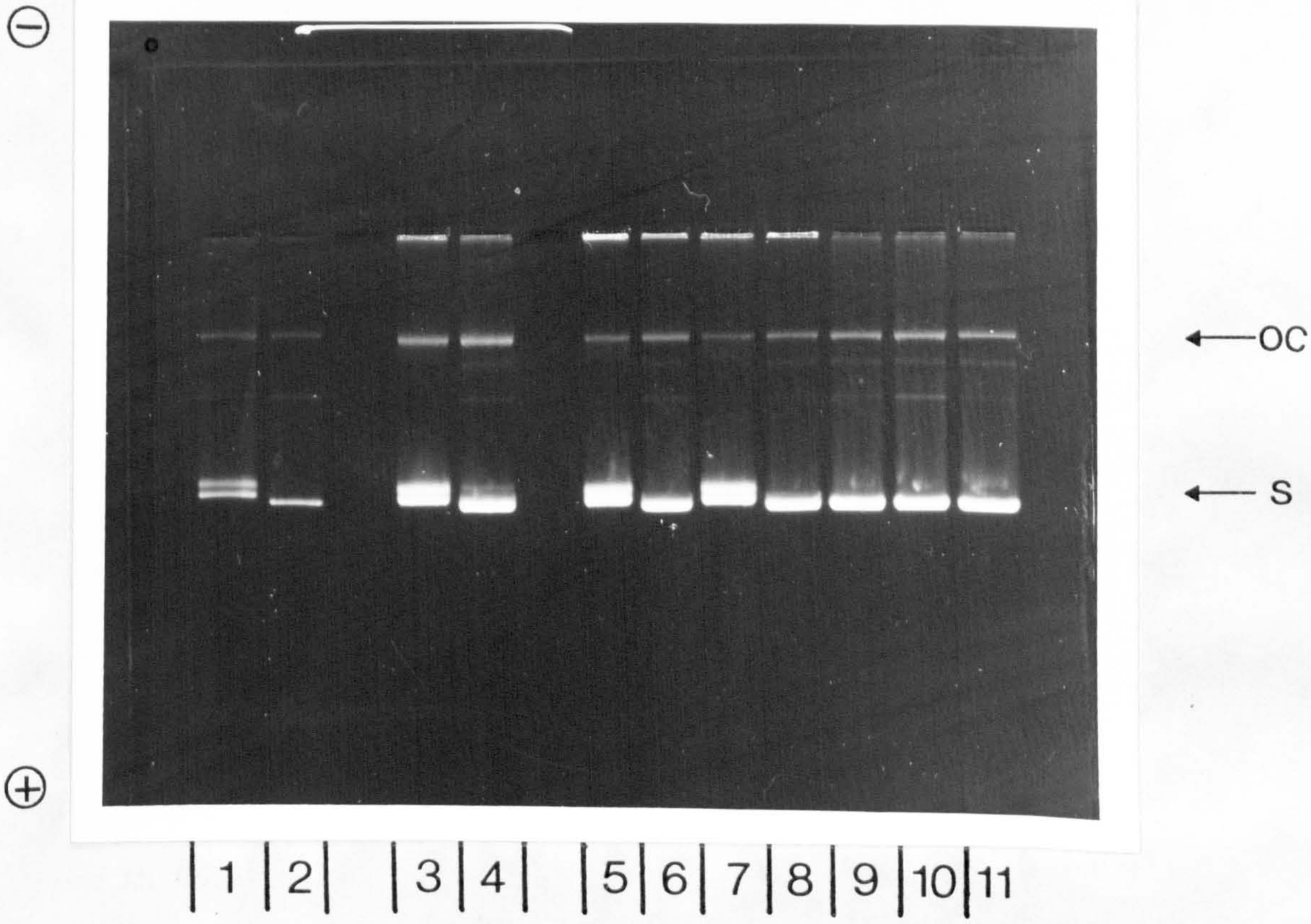
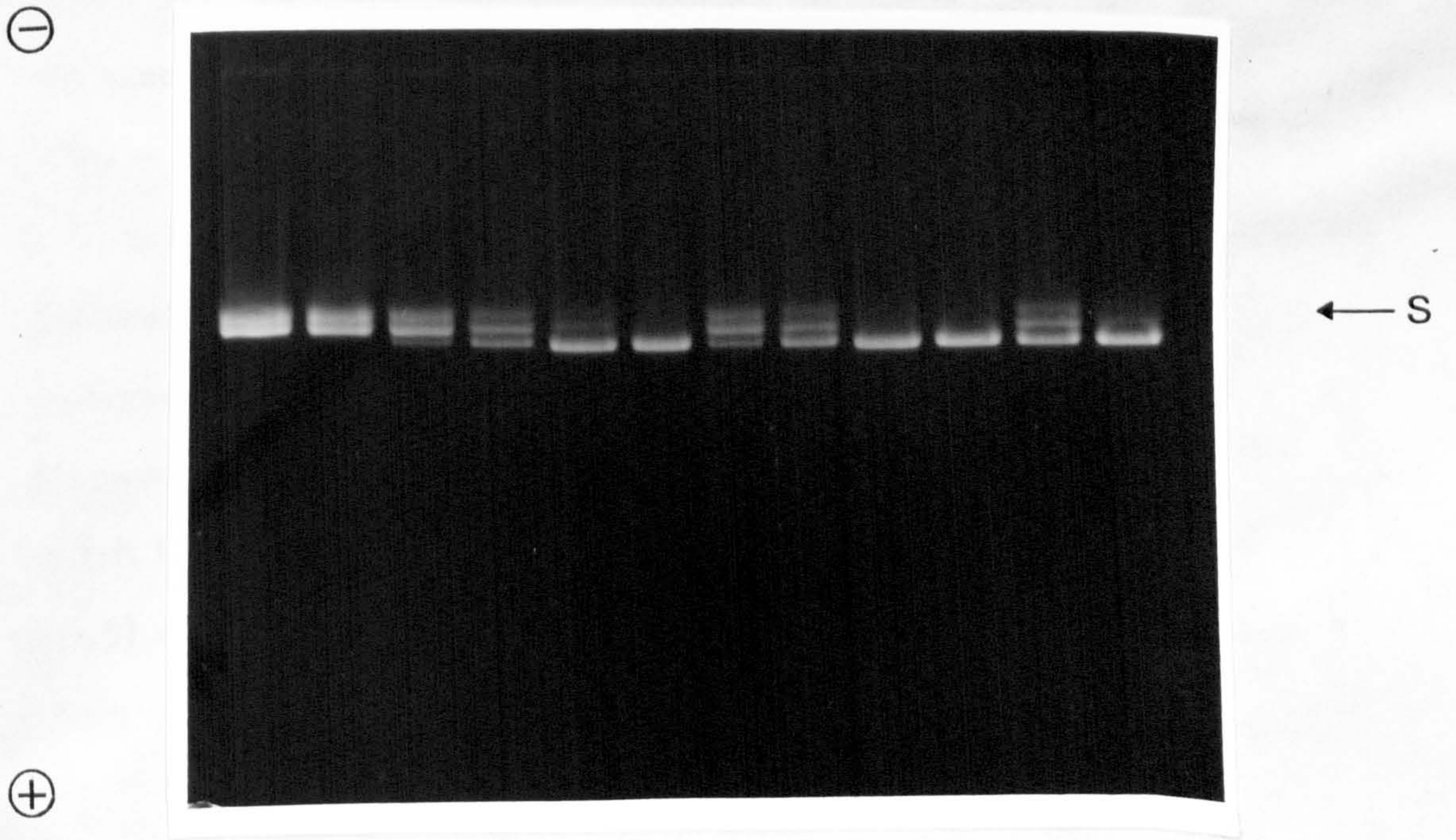


Figure 52



gradient analysis is also definitive (Henry and Knippers, 1974), but cumbersome when screening many fractions.

Endonuclease activity was detected in fraction 81 (Fig 49). The coincidence with DNA polymerase was probably fortuitous, since Chang (1978) extensively purified DNA polymerases I and II from S.cerevisiae, and did not report an associated endonuclease. However, it is a feature of other DNA polymerase purifications that endonuclease copurifies in many steps, as was shown by Méchali and De Recondo (1975) for DNA polymerase from regenerating rat liver.

The activity eluting in the region of fraction 81 was probably the same as an activity observed by Pinon and Leney (1975) using DEAE-Sephadex chromatography. The activity was not characterised by these authors. Endonucleases B and C (Pinon and Leney, 1975) both occur in the flow-through from DEAE-Sephadex, and probably correspond to the activity seen at fraction 19 in figure 44. The yeast nuclear enzyme, endonuclease α, observed by Bryant and Haynes (1978) has in common with fraction 81 its binding to and elution from DEAE-cellulose, and also its ability to introduce nicks into supercoiled DNA molecules (Figure 49). In this latter respect fraction 81 differs from endonuclease A (Pinon, 1970). Since the activity was found to be not thermosensitive, using a substrate which corresponded as closely as possible to the in vivo template, it was not further characterised. The precipitation phenomenon associated with fraction 81 upon addition of 7mM $MgCl_2$ (and seen with further purified yeast DNA polymerase I, 6.3.3) may be related to the ionic strength dependent precipitation of phosphoproteins from calf-thymus nuclei observed by Gershey and Kleinsmith (1969).

From the same DEAE-cellulose gradient (Figure 44) an activity (fraction 75), was discovered which caused a topological change in the structure of supercoiled plasmid DNA. This activity was not thermosensitive when assays were analysed on agarose gels.

5.4. CONCLUSION.

Attempts to rectify the cdc7.4 mutation by the supply of a variety of exogenous metabolites were unsuccessful. The concentrations used (0.1M), should have been sufficiently high to allow some penetration into the cell (Town et al, 1976). Total DNA polymerase, DNA-dependent ATPase and SAM synthetase activities were shown to be normal in cdc7.4, as were all three RNA polymerases.

Of considerable interest however, the replication of the yeast 2 μm plasmid was shown to be under the control of the CDC gene products. In this respect it corresponded to nuclear DNA replication, and was distinct from mitochondrial DNA replication. Thus the 2 μm plasmid can be used as a model replicon to study the events during the initiation of nuclear DNA replication. For this reason, an improved method of preparation of the 2 μm plasmid was developed.

An endonuclease activity from cdc7.4 which cofractionated with DNA polymerase on DEAE-cellulose chromatography was shown to function *in vitro* at the nonpermissive temperature (38°C). An activity causing a topological change in supercoiled DNA was also shown not to be temperature sensitive.

CHAPTER SIX.

In vitro DNA synthesis.

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In vitro DNA synthesis.

6.1. Introduction.

Prokaryotic systems capable of *in vitro* DNA synthesis have been discussed in detail in 1.5. In eukaryotes, *in vitro* DNA synthesis has proved to be much more difficult to achieve. The defined systems studied so far have been capable of various steps in DNA elongation, but not initiation of DNA synthesis.

Apparent initiation has been observed in very crude systems involving nuclear transplantation (1.4.1.), or addition of cytoplasmic extracts from proliferating cells to resting nuclei. Resting frog liver nuclei have been stimulated into DNA synthesis by extracts from yeast cdc mutants (Jazwinski and Edelman, 1976) and from temperature-sensitive mammalian cell cycle mutants (Floros *et al*, 1978), when such extracts were prepared from cells grown at permissive, but not restrictive temperatures.

Much attention has been given in the last few years to the replication of eukaryotic viral chromosomes *in vitro*. De Pamphilis *et al* (1978) reviewed the synthetic abilities of five subcellular fractions from SV40 infected CV-1 monkey cells, including cell lysates, washed nuclei, nuclear extracts, SV40 nucleoprotein complexes and replicating SV40 chromosomes. These extracts were capable of performing various elongation, separation and termination functions, and their subsequent analysis has provided much information about these processes, particularly regarding the formation of Okazaki pieces in eukaryotes. However, none of these fractions was capable of initiation. The same is true for a soluble replication system using nuclear extracts from adenovirus infected HeLa cells (Horwitz *et al*, 1978). Temperature-sensitive mutants defining 3 adenovirus genes required for replication have been isolated, and the use of this *in vitro* system identified

the defect in one mutant, H5ts125, as being in a 72KMW single-strand DNA binding protein (Horwitz, 1978), which could be complemented by the addition of wild-type DNA binding protein (Kaplan et al, 1979).

In yeast, simple in vitro DNA synthesising systems have been difficult to obtain. The toluene-treated whole cell system of Hereford and Hartwell (1971) was shown subsequently to have synthesised only mitochondrial DNA (Banks, 1973). The best studied system has been the osmotically-shocked spheroplast system of Oertel and Goulian (1977). The system synthesised mostly mitochondrial DNA in ρ^+ strains, so ρ° strains were necessary to study nuclear DNA replication. The replicated DNA consisted of short 4S fragments after very short (30 seconds) pulses of (α-^{32}P)-dTTP, which grew to 6-8S with longer pulse times, reaching a maximum of 15S. Little incorporation into high molecular weight DNA was seen. Density labelling experiments with BrdUTP instead of dTTP, suggested that only 20% of the product represented strands fully synthesised in vitro, the remainder being due to addition to pre-existing strands. RNA priming of the nascent fragments was indicated by the isolation of small quantities of ^{32}P-labelled $2^1(3^1)$-rNMPs following labelling of DNA with (α-^{32}P)-dTTP, and treatment of the DNA with alkali. Oertel and Goulian (1979) described an in vitro system using ether permeabilised yeast cells, which produced 95% mitochondrial DNA in ρ^+ strains.

Johnston (1979) produced a concentrated yeast lysate system, based on the cellophane disc method of Schaller et al (1972), which appeared to show more extensive synthesis than the Oertel and Goulian (1977) spheroplast method, and also synthesised predominantly nuclear DNA. Again synthesis proceeded via low molecular weight intermediates which did not mature into high molecular weight DNA.

Three approaches were made in the work described in this chapter. Initially, attempts were made to use osmotically-swollen spheroplasts. A second method involved the fusion of protein-filled liposomes with

cdc7.4 spheroplasts. This technique has been used extensively for the introduction of a wide variety of active materials into cells (review, Poste et al, 1976). The third approach was the development of a reconstituted system from partially purified components, using the plasmid pJDB219, containing the yeast 2 μm plasmid DNA sequences (Beggs, 1978) as a model replicon.

Convenient methods for the purification of yeast DNA polymerases I and II (Chang, 1977) and yeast RNA polymerases I, II and III (Valenzuela et al, 1979) have now been reported. Plevani and Chang (1977) have used all three RNA polymerases to prime DNA replication by DNA polymerase I, but not DNA polymerase II, on single-stranded DNA. From comparison of the properties of yeast DNA polymerases I and II, Chang (1977) has suggested that the replicative enzyme in yeast is DNA polymerase I, which appears to correspond to mammalian DNA polymerase α (Holmes and Johnston, 1975). The yeast RNA polymerases appear to correspond functionally to the similarly numbered RNA polymerases of higher eukaryotes, but differ from them in their α-amanitin sensitivity (Chambon, 1975). The specific transcription of rRNA genes by yeast RNA polymerase I (Holland et al, 1977; Van Keulen and Retel, 1977), and its specific localization in the nucleolus (Sebastian et al, 1973) suggests that this enzyme is responsible for rRNA synthesis in vivo. The differential α-amanitin sensitivities of the three enzymes has also proved valuable for the elucidation of their in vivo functions. Thus RNA polymerase II, with the greatest sensitivity to the inhibitor, was shown to synthesise heterogeneous nuclear RNA (Reeder and Roeder, 1972). Likewise, Weinmann and Roeder (1974) showed that the synthesis of tRNA and 5S RNA species in mouse myeloma cells was inhibited to the same extent by α-amanitin as solubilised RNA polymerase III activity.

In this work, attempts were made to use partially purified yeast RNA polymerases I, II and III to prime DNA synthesis on a supercoiled DNA template by yeast DNA polymerase I. Other putative components of

the replication apparatus (1.5.6.) were also partially purified, including a single-strand DNA specific binding protein (Herrick and Alberts, 1976) and a DNA topoisomerase (Champoux, 1978).

6.2. Methods.

6.2.1. Production of ρ^0 strains.

The method was that of Clark-Walker (1972). Small colonies growing on YP agar plates (0.5% yeast extract, 1% Difco peptone, 0.3% KH_2PO_4, 4% glycerol, 0.1% glucose) in the presence of ethidium bromide were selected.

6.2.2. Preparation and use of liposomes.

Liposomes were formed using 7 μmoles phosphatidyl choline, 2 μmoles cholesterol and 1 μmole dicetyl phosphate. Individual lipids were stored at 4°C in $CHCl_3$:MeOH(2:1). After mixing, the solvents were removed by a stream of nitrogen. 0.25-1.0ml of protein solution (in ice) was added to the lipids, and the mixture was shaken for about 1 minute until no lipid remained on the wall of the tube. Each mixture was then sonicated for 2x5 second intervals using the microprobe on an MSE sonicator, keeping the tube chilled in ice. The resulting mixture of vesicles, many of which were multilamellar (Poste et al, 1976), were stored in ice and used within 1-2 hours.

Solid sorbitol was added to the liposomes to give a final concentration of 1M. 0.1ml of liposomes was mixed with 0.1ml of spheroplast suspension at $4x10^8$ cells/ml in M-1 medium (YESD containing 1M sorbitol) followed by 0.2ml 40% PEG in M-medium. Chloramphenicol was added to a final concentration of 50 μg/ml. The addition of PEG caused the agglutination of spheroplasts as viewed by light microscopy. The mixture was incubated at 38°C for 30 minutes to allow fusion, and then diluted with 2ml M-1 medium containing 10 μCi/ml 6-^{3}H uracil and incubated for specified times. Samples for DNA and RNA synthesis were processed as described in 2.6.1 and 2.6.2.

6.2.3. Preparation of yeast DNA polymerases.

DNA polymerases were purified from 20-25g wet weight log-phase cdc7.4 cells grown in YEPD medium, or from 1kg commercial yeast slab (UCL Refectory). All steps were performed at 0-4°C. Cell extracts were prepared in a Braun homogenizer (2.12) or in a Manton-Gaulin homogenizer, model 15M-8BA. One unit of DNA polymerase activity was the amount which caused the incorporation of 1nmole dTMP into acid precipitable material at 30°C in 1 hour using an activated DNA template (2.14). The method was that of Chang (1977), with the following modifications. 0.5% protamine sulphate was used for precipitation of nucleic acids: this did not precipitate DNA polymerase activity. The phosphocellulose step was performed by slurrying the dialysed extract with an equal bed volume of phosphocellulose P11, stirring at 4°C for 1 hour. The phosphocellulose was washed with 0.2M KCl, and DNA polymerase activity was eluted batchwise with 0.6M KCl. The DEAE-cellulose column was 35x2.7cm, and a one litre 0-0.5M KCl gradient was used. Active fractions containing DNA polymerases I and II (Figure 59) were pooled, precipitated with 60% ammonium sulphate, resuspended and dialysed, then chromatographed on denatured DNA-cellulose columns (10x1.8cm). The crude extract contained 0.5 units DNA polymerase activity/mg protein, ten fold lower than the extracts reported by Chang (1978). Enzyme activity was consequently lower throughout the purification. After DEAE-cellulose, DNA polymerase I and II fractions contained 54.0 units/mg and 7.0 units/mg protein respectively. Consequently further purification was restricted to denatured DNA-cellulose chromatography, during which excessive loss of activity was found (final specific activities were 19.0 units/mg and 4.0 units/mg respectively).

6.2.4. Preparation of yeast RNA polymerases.

RNA polymerases were purified from 300g wet weight Youngs Brewery yeast strain 1318, or from 1kg commercial yeast slab, by the method of Valenzuela et al, (1978). The assay for RNA polymerase was described in 2.13.2. One unit of activity was the amount which caused incorporation of 1nmole rUMP into TCA precipitable material in 10 minutes at 30°C. Column dimensions were adjusted to suit the extract volumes corresponding to those used by Valenzuela et al, (1978). The method involved seven chromatographic steps which will be summarised here. An initial phosphocellulose step separated RNA polymerase I (bound) from RNA polymerases II and III (flow through). RNA polymerase I was then purified by DEAE-Sephadex A25 chromatography. RNA polymerases II and III were further purified by polyethyleneimine precipitation, and then separated on a DEAE cellulose column. The flow through activity was RNA polymerase III, which was further purified by DEAE-Sephadex A25 and denatured DNA-cellulose chromatography. RNA polymerase II which bound to DEAE-cellulose, was subjected to another phosphocellulose step. The final sucrose gradient step used by Valenzuela et al, (1978) was not employed, since the fractions obtained were nuclease free in 20 minute assays at 37°C on agarose gels (2.17).

6.2.5. Purification of single-strand DNA binding protein.

A crude extract from 20g wet weight A364A cells grown in YEPD-AU (2.3) was prepared as described in 2.12, except that the buffer was 20mM Tris-HCl pH8.0, 0.5mM EDTA, 10mM β-mercaptoethanol; 1mM PMSF; 1% DMSO (Buffer A). The crude extract was prepared in Buffer A containing 2M NaCl. The dialysed PEG supernatant (2.12) was loaded directly onto a 2x15cm native DNA cellulose column coupled to a 2.5x12cm denatured DNA cellulose column (Herrick and Alberts, 1976). After washing with 1 litre Buffer A containing 50mM NaCl, the columns were separated, and the denatured DNA cellulose column eluted with a 0.05-2M

NaCl gradient in Buffer A. Fractions were collected and analysed by SDS-polyacrylamide gel electrophoresis (2.10).

6.2.6. Southern transfer of denatured DNA to nitrocellulose and hybridization of RNA transcripts.

The method was that of Southern (1975) as described by J. Williams and R. M. Kay (personal communication). All solutions were autoclaved where possible, otherwise 1 drop of diethylpyrocarbonate was added and the solutions incubated at 65°C for 30 minutes. After the transfer, the millipore sheet (Schleicher and Schull BA85) was removed, air dried, cut into strips corresponding to gel tracks and baked in a vacuum oven at 90°C for 2 hours.

The baked strips were incubated for 3 hours at 65°C in a sandwich box containing 50ml prehybridization buffer (3xSSC, 2mg/ml BSA, 2mg/ml Ficoll 400K, 2mg/ml polyvinyl pyrrolidone (Sigma), 0.1% SDS) with gentle shaking. This solution was replaced with 50ml degassed hybridization buffer at 65°C (as prehybridization buffer, +50 μg/ml heat denatured calf thymus DNA). ^{32}P-RNA transcripts (10^7cpm) after gel filtration through Sephadex G-200 to remove (α^{32}-P)-rUTP were mixed with 7ml portions of hybridization buffer, and each incubated with a nitrocellulose strip in heat-sealed polythene bags for 60 hours at 65°C.

6.2.7. In vitro DNA synthesis assay mix.

50 μl assays contained 50mH Tris-HCl, pH8.0; 3mM ATP; 300 μM CTP, GTP, UTP; 60 μM dATP, dCTP, dGTP; 20 μM dTTP; ^{3}HdTTP (1000cpm/pmole); 1mM DTT; 6.5mM $MgCl_2$; 20 μg/ml pJDB219 plasmid DNA. Assays were stopped by the addition of 0.5ml 0.1M sodium pyrophosphate and DNA precipitated by 3ml ice-cold 5% TCA. After 15 minutes in ice, reactions were filtered through Whatman GF-C 2.5cm circles and counted in Brays scintillation fluid (2.6).

6.3 RESULTS and DISCUSSION.

6.3.1. Osmotically-shocked spheroplast method.

To avoid interference from mitochondrial DNA replication, which was known to continue unaffected in cdc7.4 at the restrictive temperature (3.3.2.1; 5.3.4.1), a ρ^{o} derivative of cdc7.4 (H201.14.4) was obtained by ethidium bromide treatment (6.2.1; Clark-Walker, 1972). Several ρ^{-} strains were labelled overnight with 10 μCi/ml 6-^{3}H uracil, then mixed with portions of a culture of cdc7.4(ρ^{+}) which had been labelled overnight with 0.25 μCi/ml 2-^{14}C uracil. Lysates were prepared from the mixtures and caesium chloride density gradients of the DNA were run (2.15.1). Figure 5^{3} shows a gradient of one such strain, which convincingly lacked mitochondrial DNA. This cdc7.4 ρ^{o} strain was used in the osmotically-shocked spheroplast system.

It had been shown that the leakage of intracellular material from yeast spheroplasts was dependent on the molarity of the sorbitol present as osmotic support (Kuo and Yamamoto, 1975). An experiment was performed to determine the leakiness of spheroplasts prepared by the glusulase method (2.9.1). The results in Figure 54 show that below 0.8M sorbitol spheroplasts began to leak OD_{260} absorbing material. Moreover, the leakage was almost immediate, since zero time readings did not differ markedly from 2hour incubated samples, which also implied that there was not extensive disintegration of spheroplasts during this period, at low sorbitol concentrations.

Since Kuo and Yamamoto (1975) also observed that the leakage of an intracellular enzyme (α-glucosidase) also followed the OD_{260} absorbing material leakage, it seemed possible that spheroplasts suspended in sorbitol in the 0.3 to 0.6M range might be permeable to exogenously supplied nucleotides and proteins. Such an experiment was performed upon cdc7.4 ρ^{o} cells which had been arrested at the cdc7.4 block by incubation at 38°C for 2 hours. Assays for DNA synthesis were performed at 23°C

Figure 53: Analysis of a cdc7.4ρ^{o} strain by caesium chloride density gradient centrifugation.

A 10ml culture from a small cdc7.4 (H201.14.4) colony growing after ethidium bromide treatment (6.2.1) was labelled overnight with 10 μCi/ml 6-^{3}H uracil. A 5ml portion of cdc7.4ρ^{+} which had been labelled overnight with 0.25 μCi/ml 2-^{14}C uracil was added and a mixed lysate prepared and centrifuged as in 2.15.1. (O), ^{3}H; (●), ^{14}C.

Figure 54: Leakage from osmotically swollen spheroplasts.

The cdc7.4ρ^{o} strain obtained in Figure 53 was grown to mid-log phase in YEPD-AU, and spheroplasts were prepared using β-glucuronidase as described in Figure 16. The spheroplasts were split into ten 1ml portions, centrifuged and resuspended in duplicates in 0.2M, 0.4M, 0.6M, 0.8M and 1M sorbitol. 1 portion was centrifuged immediately and the OD260nm of the supernatant was measured (O). The other portions remained in suspension for 80 minutes prior to OD260nm measurement (●).

Figure 53

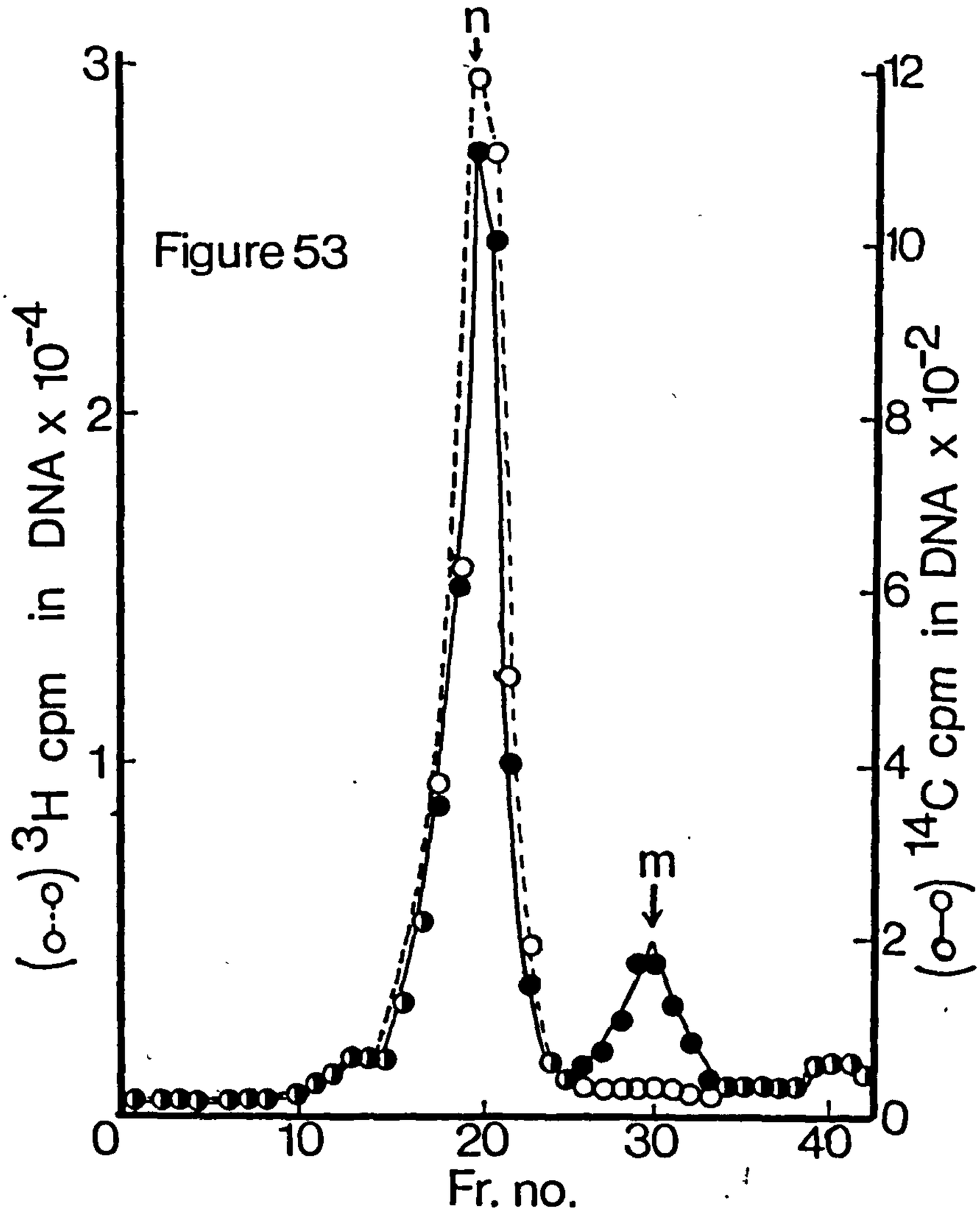


Figure 54

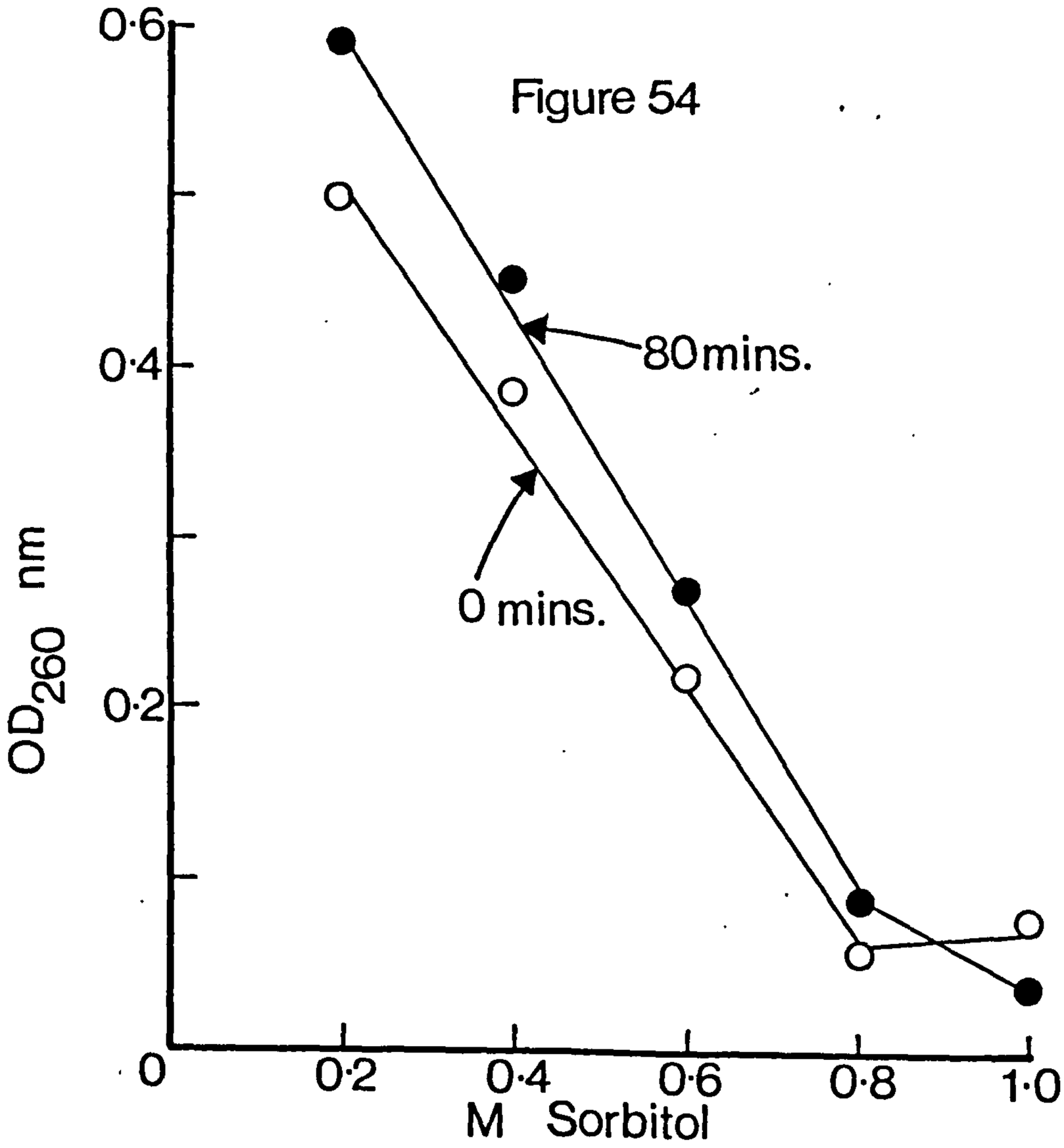
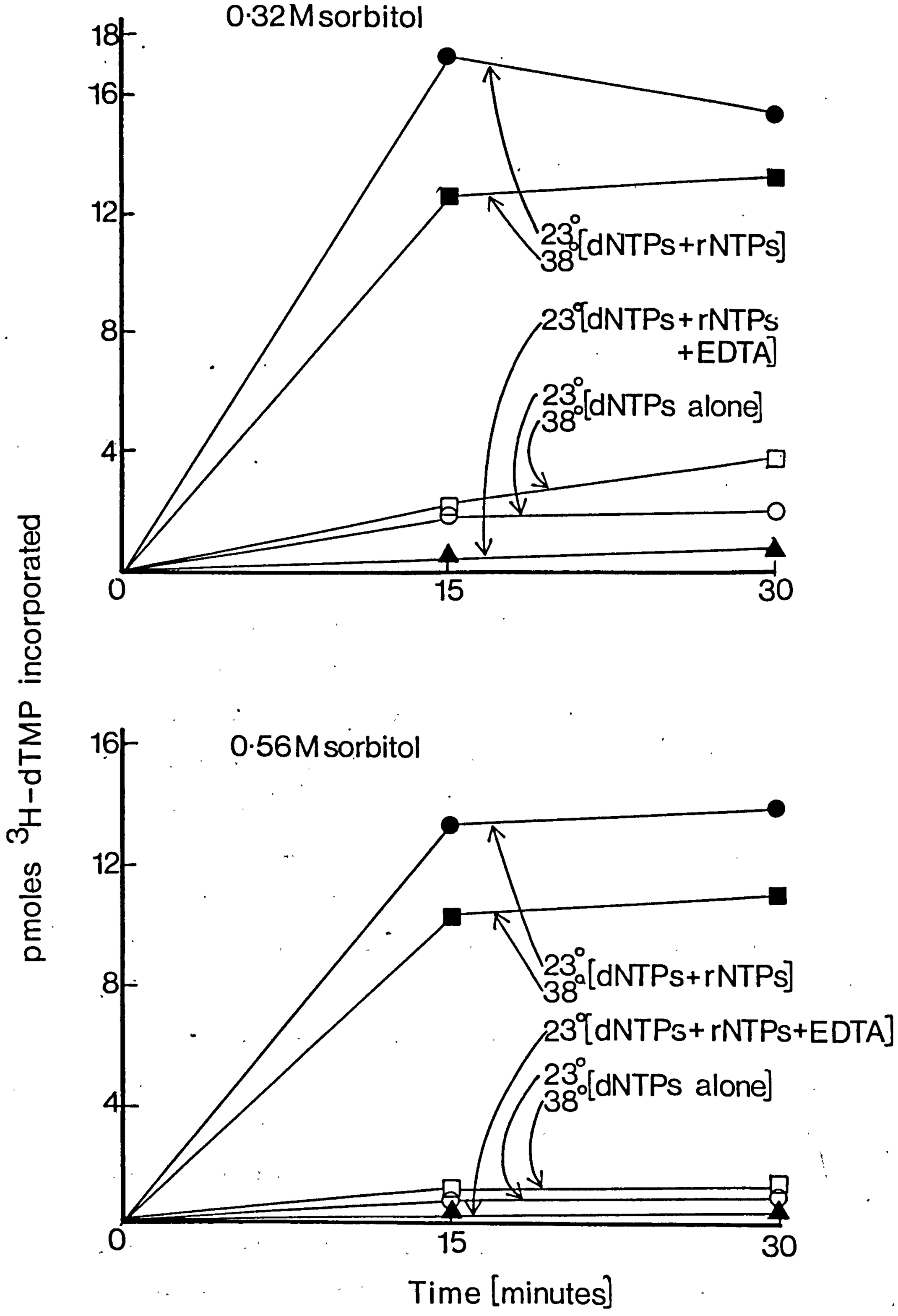


Figure 55: DNA synthesis in osmotically swollen spheroplasts using exogenous nucleoside triphosphates.

A 10ml exponential culture of cdc7.4 β° grown in YEPD-AU medium was shifted to 38°C for 2 hours. After 65 minutes, to allow all S phases underway to be completed (5.3. 4.1), 0.1M hydroxyurea was added to prevent further DNA synthesis (Slater, 1973; Hartwell, 1977). Spheroplasts were produced as described in 2.9.3, except that the temperature was 37°C and 0.1M hydroxyurea was present. After washing in ice-cold 0.8M sorbitol, 5mM EDTA, 0.1M hydroxyurea, the spheroplasts were resuspended ice-cold 0.8 and 1.4M sorbitol at 5×10^8 spheroplasts/ml. 100µl incubations were performed at 23°C and 38°C using 40µl of the spheroplasts suspensions and containing 100mM Tris-HCl, pH7.8; 166µM dATP, dGTP, dCTP and dTTP; 8.3mM $MgCl_2$; 3.5mM DTT; 33µCi/ml methyl-^{3}H dTTP (55cpm/pmole); 8.3 g/ml BSA. Duplicate mixes also contained 1mM rATP, rGTP, rCTP and rUTP. 25µl samples were removed at 0, 15 and 30 minutes after mixing and processed as described in 2.13. Final sorbitol concentrations were A, 0.32M and B, 0.56M. Open symbols, minus rNTPs; solid symbols, plus rNTPs; (○), 23°C; (●), 38°C.


0·32 M sorbitol
18
16
12
8
4
0
15
30
23°[dNTPs+rNTPs]
38°
23°[dNTPs+rNTPs+EDTA]
23°[dNTPs alone]
38°
pmoles 3H-dTMP incorporated
0·56 M sorbitol
16
12
8
4
0
15
30
23°[dNTPs+rNTPs]
38°
23°[dNTPs+rNTPs+EDTA]
23°[dNTPs alone]
38°
Time [minutes]

and 38°C in the presence or absence of rNTPs, and at final sorbitol concentrations of 0.32M and 0.56M. The results are shown in Figure 55A and B.

The most remarkable feature of the figures is the stimulatory effect of rNTPS. The addition of dNTPS alone did not allow significant synthesis above a control incubation containing 18mM EDTA. Synthesis in the incubations appeared to be complete at 15 minutes, and 23°C incubated samples incorporated more radioactivity (~20%) than corresponding 38°C incubations. The result seemed promising, except that it showed that the treatment used to arrest the cells at the cdc7 block was unsatisfactory, possibly due to the brief chilling during the spheroplast preparation.

However, ten attempts to reproduce and extend the results from this system were unsuccessful. Variations were performed using Arthrobacter and glusulase derived spheroplasts (2.9), various buffer systems, the addition of nonidet LE35 detergent, and upon blocked and unblocked cells. Microscopic examination of the spheroplasts following each of these treatments showed that the integrity of the spheroplasts was related to their treatment. Arthrobacter derived spheroplasts were much more fragile than glusulase produced ones, such that suspension in low sorbitol concentrations as used in Figure 55 was found to cause osmotic lysis. However, despite the lack of cell wall and outer membrane, cell contents were frequently seen to be clumped together. It is possible that the precise conditions used for Figure 55 were sufficient to cause lysis without disruption of cell contents, which could not be reproducibly obtained. Consequently, this experimental approach was abandoned.

6.3.2. Liposome fusion experiments.

The basic approach involved the fusion of protein-filled liposomes with yeast spheroplasts using 20% polyethyleneglycol. To avoid triggering

DNA replication in cdc7.4 spheroplasts held at the restrictive temperature, it was necessary to have all the components preincubated at 38°C before fusion. Initially, liposomes filled with crude protein extracts from wild-type cells were fused with spheroplasts of exponential cdc7.4 cells, and complementation was assayed by measuring DNA synthesis at 38°C. Liposome fusion experiments were always performed in the presence of 50 μg/ml chloramphenicol to eliminate possible effects due to bacterial contamination since the liposomes were not prepared under sterile conditions (6.2.2). The order of addition to prepared spheroplasts was always: i) liposomes, ii) any other additions (buffer, etc), iii) 40% PEG.

6.3.2.1. Growth of spheroplasts.

It was essential to prove that spheroplasted cdc7.4 cells retained the ability to make DNA at 23°C but not at 38°C. A culture of log-phase cdc7.4 (DE200.1.3) cells was spheroplasted using Arthrobacter enzyme (2.9.3) and the resulting spheroplasts were incubated in YESD medium containing 1M sorbitol (M-1 medium) and 10 μCi/ml 6-^{3}H uracil. DNA and RNA synthesis was monitored at 38°C and 23°C as described in 2.6. The results in Figure 56 showed that at 23°C DNA and RNA synthesis continued exponentially for at least 6 hours. At 38°C, DNA synthesis reached a plateau after 2 hours, and RNA synthesis ceased at 4 hours, followed by a loss of label, presumably due to cell death (3.3.1.1).. Thus it seemed that 4 hours was optimal for observing the difference in DNA synthesis between 23°C and 38°C grown spheroplasts. The DNA synthesised in the first 2½ hours at 38°C in cdc7.4 spheroplasts was shown to be 65% nuclear and 35% mitochondrial by caesium chloride gradient centrifugation.

The optimum spheroplast concentration for radioactive incorporation was also studied. Figure 57A shows that for cdc7.4 (DE200.1.3), a plateau in RNA and DNA synthesis was reached using 2-3x10^{7} spheroplasts/ml. However, when this work was repeated using wild-type parent A364A and

Figure 56: Synthesis of DNA and RNA in spheroplasts of cdc7.4.

A 45ml exponential culture of cdc7.4 (DE200.1.3) in YESD medium was spheroplasted using Arthrobacter enzyme as described in 2.9.3, and the spheroplasts were resuspended in YESD medium containing 1M sorbitol (= M1 medium) and 10 μCi/ml 6-^{3}H uracil at a density of 6.5×10^{6} spheroplasts/ml. The suspension was split into two 10ml portions one of which was shifted to 38°C (●), the other remaining at 23°C (O) 0.5ml and 0.1ml samples were removed at the times indicated for DNA and RNA synthesis respectively. (a) DNA synthesis, (b) RNA synthesis.

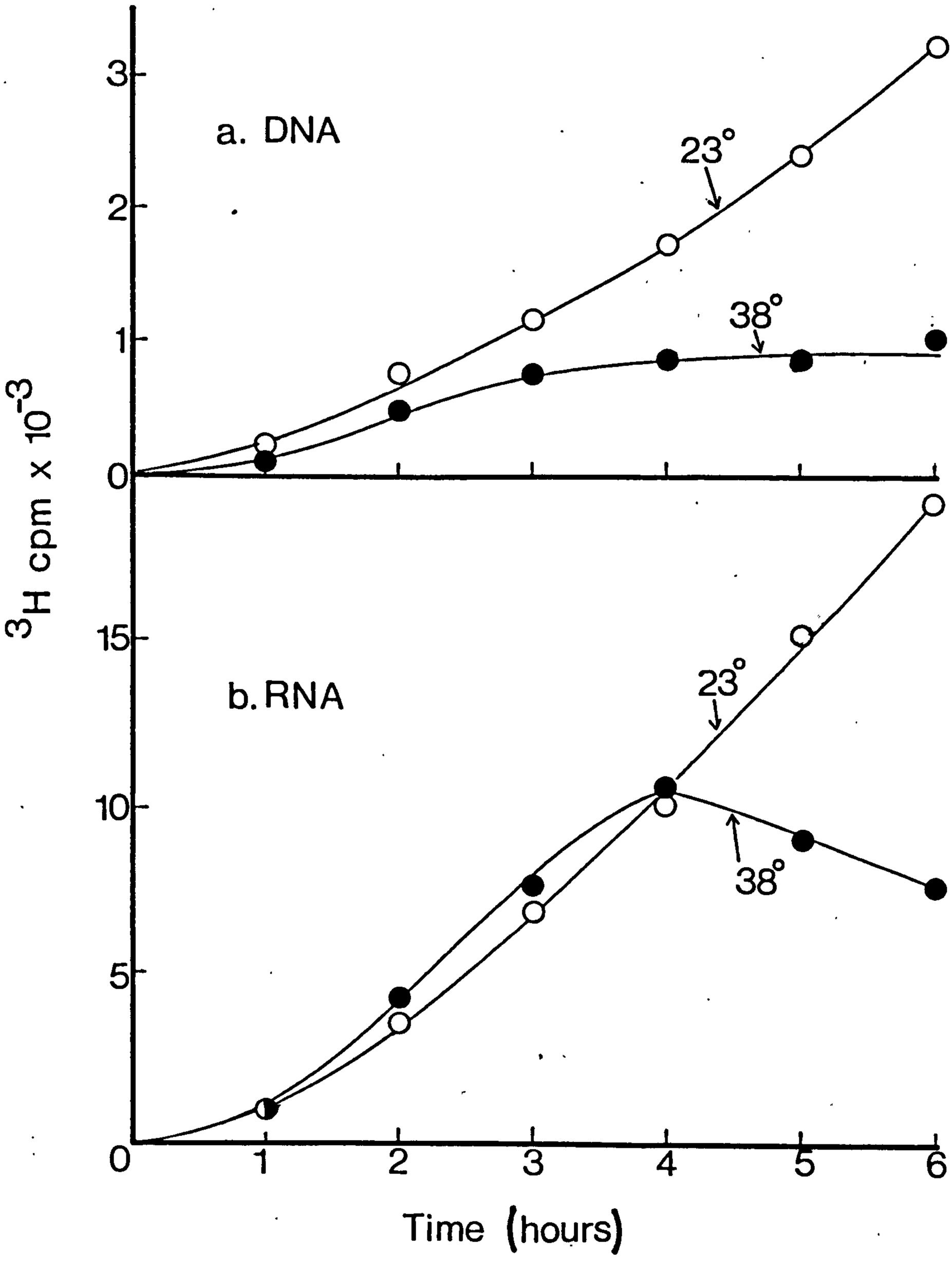

a. DNA
23°
38°
b. RNA
23°
38°
^{3}H cpm x 10^{-3}
0
1
2
3
5
10
15
0
1
2
3
4
5
6
Time (hours)

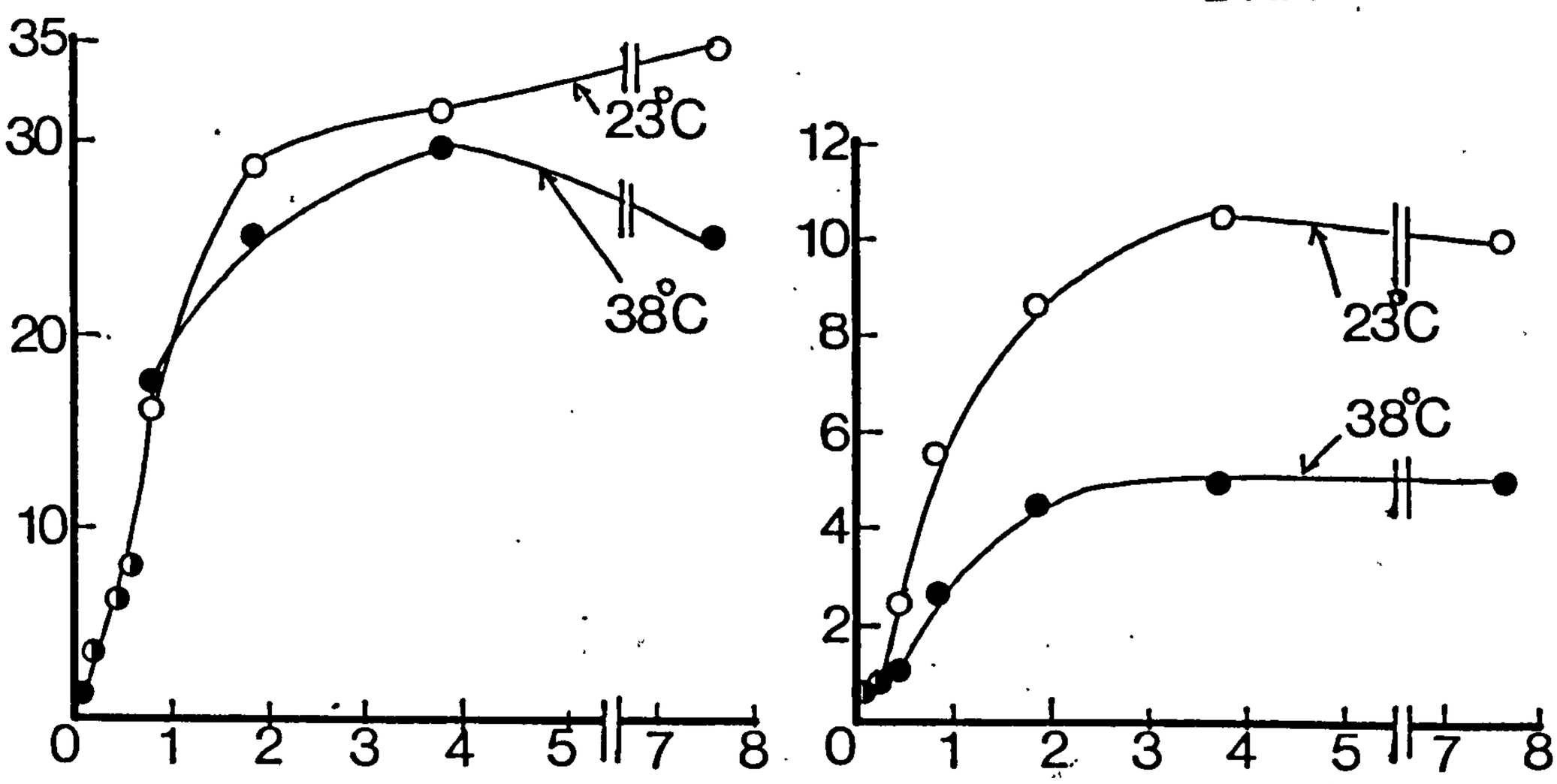
Figure 57: Effect of spheroplast density on DNA and RNA synthesis.

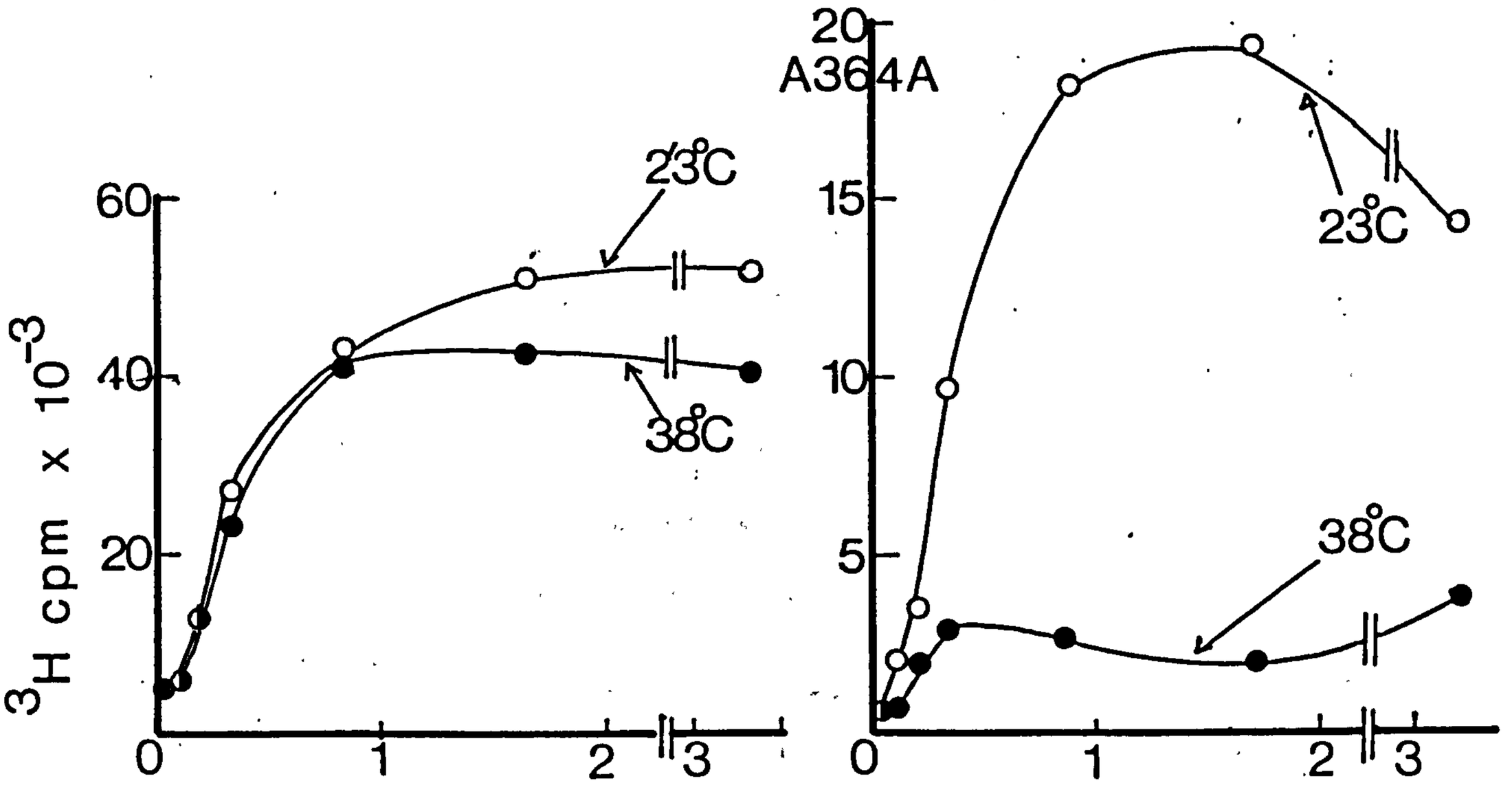
Washed spheroplasts prepared from exponential cultures of cdc7.4 (DE200.1.3), A364A and X2180-1A as in Figure 56, were resuspended at various densities in M1 medium in duplicate portions which were incubated at 23°C (open symbols) and 38°C (solid symbols). After 4 hours samples were removed for DNA and RNA synthesis as in Figure 56.

cdc7.4

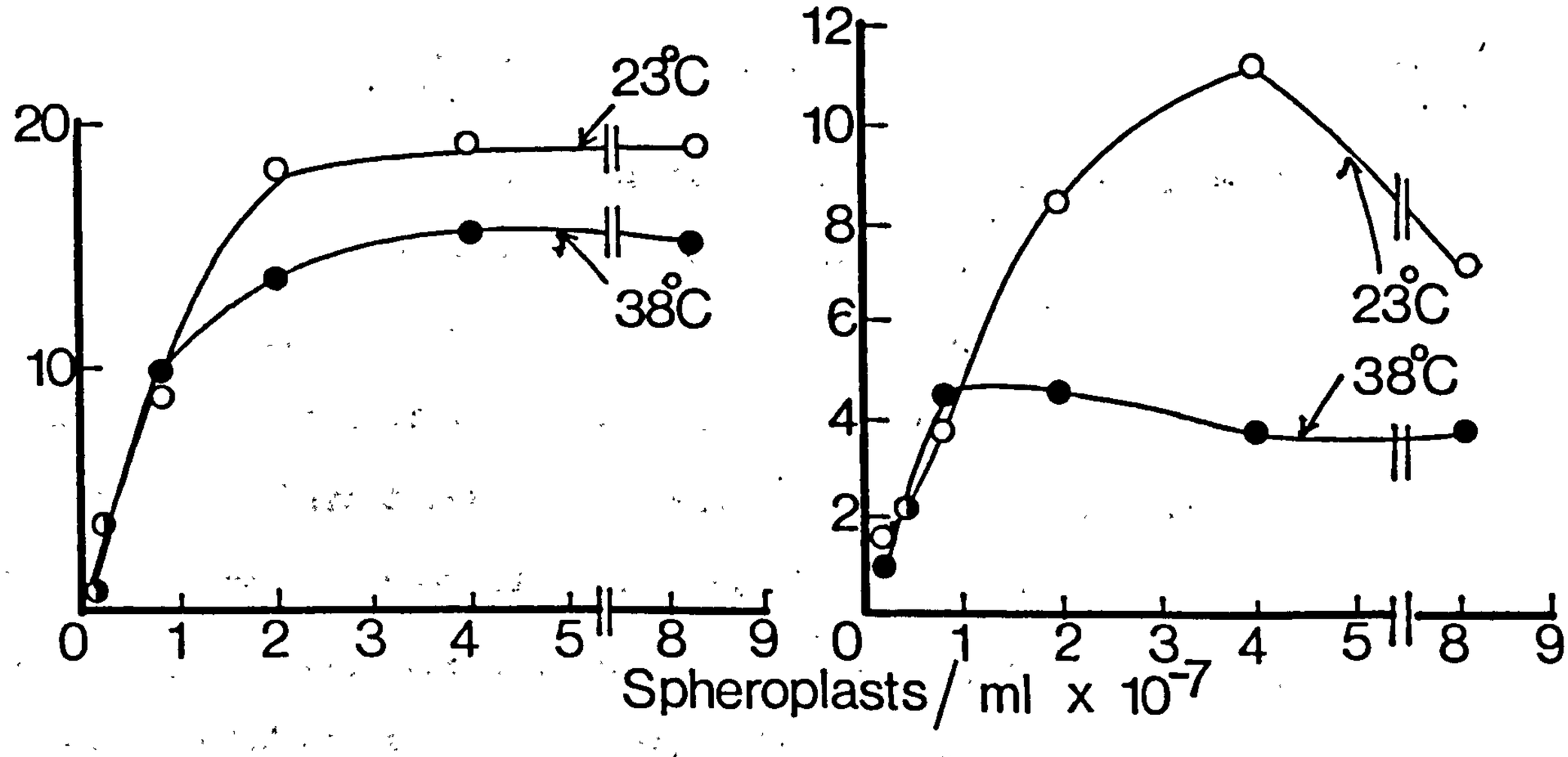
RNA

DNA





X2180-1A



Spheroplasts / ml x 10^{-7}

protrotrophic X2180-1A spheroplasts (Figure 57B and C), the results were remarkably similar to those for cdc7.4. In fact, A364A spheroplasts (Figure 57B) appeared to be more temperature-sensitive than those of cdc7.4. This observation proved to be the major problem in this approach. Clearly, it was difficult to establish a complementation assay when the defective character could not be distinguished from the wild-type phenotype. This work suggested that the stability of spheroplasts was a strain-dependent characteristic. Also, it confirmed that the use of spheroplasts held at the cdc7.4 temperature block would be necessary to prove a complementing activity.

It was observed in other experiments that Arthrobacter enzyme and glusulase derived spheroplasts were indistinguishable in DNA and RNA synthesis in M-1 medium over a 4 hour period (data not shown). Thus Arthrobacter enzyme was used throughout this work. The spheroplasts produced did not appear to regenerate cell wall or divide even after overnight incubation at 23°C in M-1 medium. Instead, they grew very large (10 x normal size). It had been reported previously that glusulase derived spheroplasts regenerated their cell walls after a short period of growth (Hutchison and Hartwell, 1967). The greater effectiveness of Arthrobacter enzyme for cell wall removal (3.3.5) may be responsible for this effect.

6.3.2.2. Effect of liposomes containing crude extracts on DNA synthesis in cdc7.4 spheroplasts.

Liposomes were charged with three crude extracts derived from log-phase A364A cells. One preparation, A, was a complete unfractionated extract (2.12). Two others, B and C, were 0-50% and 50-80% ammonium sulphate fractions of a similarly derived crude extract. The effect of liposomes charged with equal protein concentrations (9.6mg/ml) of all three preparations upon DNA synthesis in cdc7.4 spheroplasts at 38°C is shown in Table XI. The spheroplasts were produced from exponentially growing cdc7.4 cells. Control incubations included spheroplasts alone,

Table XI. Stimulation of DNA synthesis in sperhoplasts by crude extracts.

INCUBATIONS						^{3}Hcpm incorporation			
Number	Spheroplasts	Liposomes	40% PEG in M-1 medium	M-1 medium	CAP	RNA cpm	RNA stimulation	DNA cpm	DNA stimulation
1	0.2ml ($8.5x10^7$/ml)	0.2ml+3mM KPi buffer pH7.2; 1mM DTT	0.4ml	none	50 g/ml	30,360	0	4190	0
2	"	0.2ml+fraction A.	"	none	"	35,950	18%	6575	57%
3	"	0.2ml+ fraction B.	"	none	"	40,950	35%	5800	39%
4	"	0.2ml+ fraction C.	"	none	"	36,800	21%	6035	44%
5	"	none	"	0.2ml	"	30,265	-	4780	-
6	"	none	none	0.6ml	"	24,217	-	4990	-

Note: Incubations were performed at 38°C for 2½ hours.

and spheroplasts plus 20% PEG. The major control used here, upon which estimates of stimulatory activity were based was spheroplasts plus buffer-filled liposomes fused with 20% PEG (incubation 1). With this as a basis, stimulation of 44%, 57% and 39% was observed for fractions A, B and C respectively.

If this effect was in fact due to complementation, and not due to the additional stabilization of the spheroplasts by the high protein concentrations, then the stimulatory activity was distributed between the two ammonium sulphate fractions. However, stabilization is suggested by the increase in RNA as well as DNA synthesis (Table XI).

6.3.2.3 Effect of more extensively fractionated crude extracts on DNA synthesis.

A crude extract from 80g wet weight of log-phase X2180-1A cells grown in YEPD medium (2.3) was prepared and fractionated by phosphocellulose and DEAE cellulose chromatography to yield twelve fractions as described in the legend to Figure 58. After concentration of these extracts by vacuum dialysis against 3mM potasium phosphate pH7.2, 5mM β-mercaptoethanol, liposomes were prepared for each fraction as described in 6.2.2, using the maximum possible protein concentrations.

Complementation assays were performed using cdc7.4 cells which had been synchronised with α-factor and then spheroplasted and washed at 38°C. 0.1ml spheroplast suspension (3.8×10^7 cells/ml) was mixed with 0.1ml of liposomes containing protein extracts and M-sorbitol, and 0.2ml 40% PEG in M-1 medium was added. The results of two such experiments are shown diagrammatically in a histogram in Figure 58. Incorporation into DNA and RNA measured as described in 2.6.1 and 2.6.2. respectively, can only be expressed as acid-precipitable cpm since the specific activity of the 6-^{3}H uracil cannot be determined (DE200.1.3 did not require uracil for growth). The control upon which estimates of stimulation were based was spheroplasts + buffer filled liposomes + 20% PEG. Three points can be made from Figure 58 :

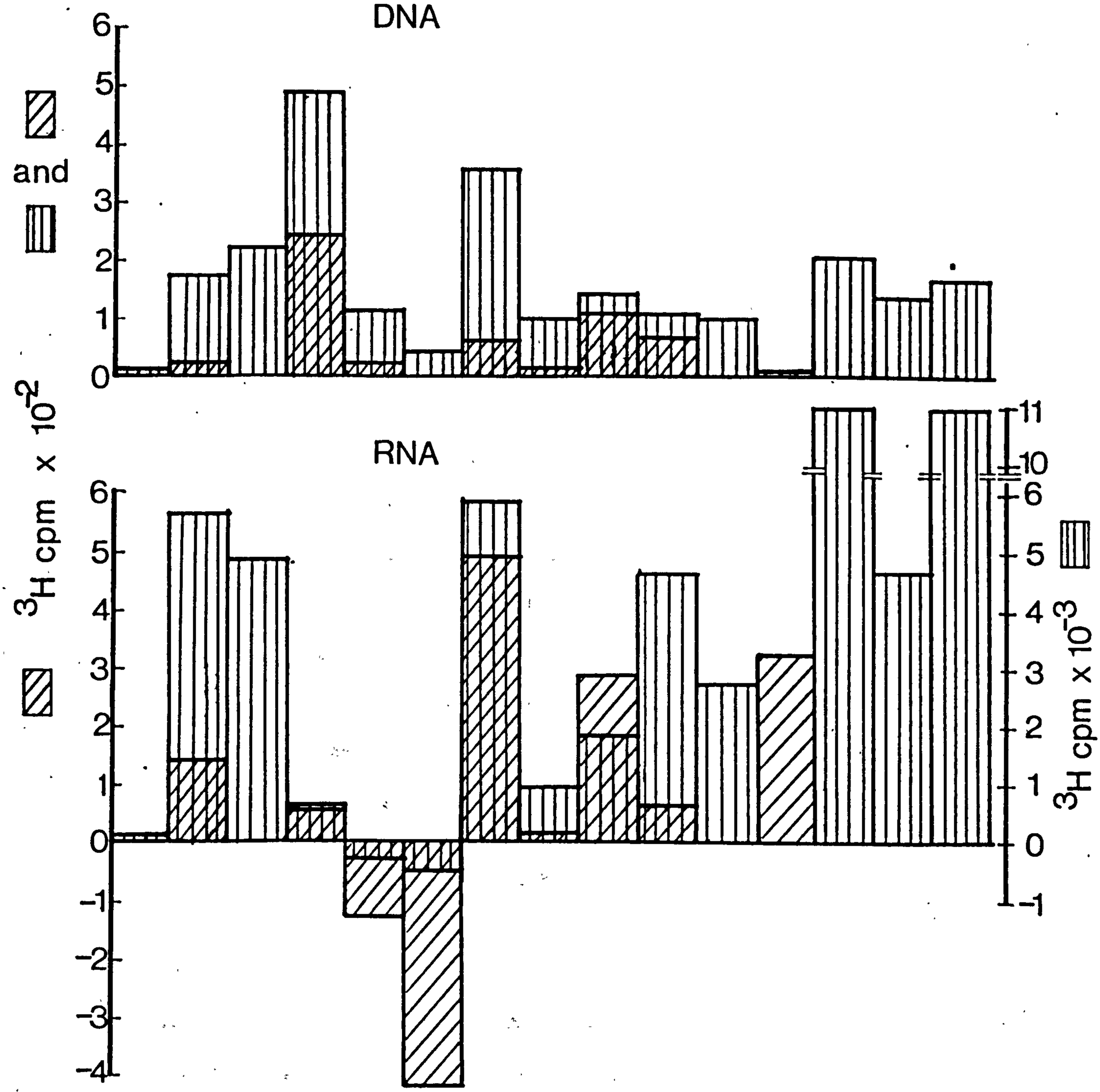
Figure 58: Liposome complementation assay using fractionated extracts.

80g wet weight of log-phase X2180-1A cells grown in YEPD medium were broken by passage (twice) through a French pressure cell at 9000-16000psi at 4°C in 10mM potassium phosphate, pH7.2; 1mM EDTA; 10% v/v glycerol; 1mM PMSF; 5mM β-met., (Buffer X). The extract (200ml) prepared as described in 2.12 was loaded onto a 42x3.3cm phosphocellulose column equilibrated in Buffer X. After washing with 500ml of Buffer X, bound proteins were bulk eluted with 500ml of Buffer X containing 500mM potassium phosphate, pH7.2, collecting three large fractions (P(I), P(II) and P(III)). The phosphocellulose flow-through (315ml) was loaded directly onto a 31x2.7cm DEAE-cellulose DE52 column equilibrated in Buffer X. The column was washed with 1 litre of Buffer X, and bound proteins were eluted with successive washes containing 50mM, 100mM, 200mM and 500mM potassium phosphate, pH7.2 in Buffer X. The first two washes were each collected as two fractions (D50(I), D50(II), D100(I) and D100(II)). With the inclusion of the phosphocellulose load (P_L), DEAE-cellulose load (D_L) and the DEAE-cellulose flow-through (D_F), this protocol yielded 12 fractions. All fractions were concentrated to 10-20ml by vacuum dialysis against Buffer X containing 5% v/v glycerol in Schleicher and Schull Ultra-Thimbles (UH100/25). Finally, the concentrated fractions were dialysed against 3mM potassium phosphate, pH7.2; 0.1mM DTT. The protein concentrations are shown in the figure. Lipsomes were charged with 0.25ml of each of these fractions as described in 6.2.2. except that the quantity of lipid used was reduced to 2.5 μmoles total.

Liposomes were fused with spheroplasts of α-factor synchronised cdc7.4 cells as described in 6.2.2. After incubation at 38°C for 3 hours 0.75ml and 0.02ml samples were removed for DNA and RNA synthesis respectively, processing as described in 2.6.

The upper and low histograms show the results for DNA and RNA synthesis respectively. The differently hatched columns give the results for two separate experiments. Incorporation is expressed relative to a control incubation of spheroplasts fused with buffer-filled (3mM potassium phosphate pH7.2, 0.1mM DTT) liposomes (zero cpm).

The assay numbers correspond to the following fractions:
1,D_F; 2,P_L; 3,D_L; 4,P(I); 5,P(II); 6,P(III); 7,D50(I); 8,D50(II); 9,D100(I); 10,D100(II); 11,D200; 12,D500; 13,Control,minus PEG; 14,Control,minus liposomes; 15,Control, minus PEG and liposomes.



Assay no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Protein conc. in liposomes mg/ml	0·1	1·6	0·7	15·5	20·8	36·0	3·3	6·0	9·7	1·0	2·0	1·4	-PEG	-liposomes	-PEG - lipos

1) Incorporation into DNA in all the tested fractions and controls was low compared with whole cells under the same conditions (data not shown).

2) 20% PEG inhibited RNA synthesis by 50-75% of control incubations without PEG (13 and 15).

3) Three fractions were of interest for their effect on RNA and DNA synthesis. Fraction P(III) assay number 6, caused a decrease in RNA synthesis below the control level (xxxxxxxx). Fractions P(I) assay number 4 and fraction DEAE 50(I) assay number 7 caused slight stimulation of DNA synthesis in one experiment. Fraction 50(I) also stimulated RNA synthesis.

Further experiments using these fractions were performed but a lack of reproducibility was found. The situation was not improved when steps were taken to eliminate PEG totally from the precipitates.

Other difficulties inherent in the system were the necessity for synchrony/of spheroplasts and for the use of low spheroplast concentrations (6.3.2.1) and the inhibitory effect of 20% PEG on RNA synthesis when using synchronised spheroplasts. As a consequence of these problems this approach was not pursued further in these studies.

6.3.3. DNA synthesis on crude extracts supplemented with DNA polymerase I.

Attempts were made to produce a concentrated crude extract from exponential cdc7.4 cells which would support replication of added pJDB219 plasmid DNA at the permissive but not at the restrictive temperature. Two crude extracts were prepared, one of which was clarified by centrifugation in a Beckman 50Ti rotor at 40K rpm for 45 minutes at 4°C, the other being treated with 10% PEG 6000 as described in 2.12. As well as increasing the OD_{280}/OD_{260} ratio, PEG precipitation in 2M NaCl removed approximately 70% of the protein from the extract. Three ammonium sulphate cuts (0-50%, 50-80% and 80-100%) were obtained from each crude extract.

These six ammonium sulphate fractions were assayed for their ability to stimulate DNA synthesis in the reaction mix described in 6.2.7., containing 10 μg/ml pJDB219 plasmid. Also mixtures in pairs were assayed. Slight incorporation (0.37 pmoles) was seen with the 0-50% fraction from the extract not treated with PEG. All other fractions gave less than 0.03 pmoles when tested individually. However, mixing the 0-50% and the 80-100% fractions from the extract not treated with PEG resulted in incorporation of 1.09 pmoles. Table XII shows that the synthesis was dependent on dNTPs, rNTPs and Mg^{2+}, but the addition of DNA polymerase I was found to be inhibitory, and the omission of plasmid DNA resulted in only a 20% reduction in total incorporation.

Table XII DNA synthesis using mixed crude extracts.

Crude extract addition	Factor omitted from assay	^{3}H-dTMP Incorporated (pmoles) (10 minutes, 33°C)
Nil	Complete	0
0-50% (5 μl)	"	0.37
80-100% (10 μl)	"	0.03
0-50% + (5+10 μl)	"	1.09
80-100% " "	rNTPS	0.27
" "	dNTPS	0.30
" "	Mg^{2+} (20mM EDTA added)	0.00
" "	pJDB219 DNA	0.89
" "	DNA polymerase I	1.53

Note: Assays used the reaction mix described in 6.2.7 containing 7 μg DNA polymerase I.

Thus the synthesis arose from the mixing of the 0–50% and 80–100% ammonium sulphate fractions and was independent of added DNA polymerase. Assays in which DNA polymerase I was omitted and the concentration of plasmid DNA was varied showed that the DNA synthesis observed was not directed by the plasmid (data not shown). The nature of the components in the two fractions was revealed by treatment with either 0.5mg/ml trypsin or 20 µg/ml (75 Worthington units/assay) micrococcal nuclease +3mM $CaCl_2$ for 16 hours at 0°C. Control incubations for each treatment included 1mg/ml trypsin inhibitor or 8mM EGTA added before the digesting enzymes. Treatments were terminated by the addition of 1mg/ml trypsin inhibitor or 8mM EGTA. Table XIII shows a complete list of treatments and their effects on the stimulation of DNA synthesis. Stimulation was measured by adding untreated 80–100% fraction to treated 0–50% fraction and vice versa.

The results indicated that the 0–50% fraction was trypsin and micrococcal nuclease sensitive, but the 80–100% fraction was only sensitive to micrococcal nuclease. Clearly, the stimulation of DNA synthesis was largely due to the addition of a nucleic acid component contained in the 80–100% fraction to enzyme and nucleic acid components in the 0–50% fraction. Addition of native or denatured DNA to the 0–50% fraction gave results which suggested that the stimulation may have been caused by single stranded DNA in the 80–100% fraction.

Despite the lack of dependency upon input plasmid DNA, this crude system was analysed extensively because it was necessary to understand the cause of the observed synthesis. Another <u>in vitro</u> system using a crude cell free extract (a 0–50% ammonium sulphate fraction) prepared from log-phase yeast cells has been reported recently (Jazwinski and Edelman, 1979), which claimed to observe DNA synthesis directed by added 2 µm plasmid DNA. The artefactual synthesis observed above must be borne in mind when interpreting the results from such crude systems.

Table XIII. The effects of trypsin and micrococcal nuclease on the 0-50% and 80-100% ammonium sulphate fractions.

No.	Fraction	Additions	Volume of treated fraction used	Untreated fraction added	Stimulation (%)
1.	100 µl 0-50% ASO_4	none	5 µl	10µl 80-100%	100
2.	"	0.5mg/ml trypsin (Sigma)	"	"	1.8
3.	"	1mg/ml trypsin inhibitor (Sigma) + 0.5mg/ml trypsin	"	"	37.1
4.	"	20 µg/ml micrococcal nuclease (Sigma) + 3mM $CaCl_2$	"	"	7.9
5.	"	8 mM EGTA + 20 µg/ml micrococcal nuclease + 3mM $CaCl_2$	"	"	104
6.	100 µl 80-100% ASO_4	none	10 µl	5 µl 0-50%	100
7.	"	0.5mg/ml trypsin	"	"	123
8.	"	1mg/ml trypsin inhibitor + 0.5mg/ml trypsin.	"	"	127
9.	"	20 µg/ml micrococcal nuclease + 3mM $CaCl_2$	"	"	1.8
10.	"	8 mM EGTA + 20 µg/ml micrococcal nuclease + 3mM $CaCl_2$	"	"	44.2

The 0-50% and 80-100% ammonium sulphate fractions were prepared with the above additions and incubated at 0°C for 16 hours. Incubations 2 and 7 were terminated by the addition of 1mg/ml trypsin inhibitor, and 4 and 9 received 8 mM EGTA. Samples were kept in ice for 1 hour then mixed and assayed as in Table XII. Stimulation is expressed relative to reactions using untreated 0-50% ASO_4 or 80-100% ASO_4 fractions alone, as appropriate.

6.3.4 Experiments on a reconstituted system from partially purified components.

6.3.4.1. DNA polymerase purification.

DNA polymerases I and II were separated on DEAE cellulose DE52 by the method of Chang (1977) as described in 6.2.3, using the assay described in 2.14. The separation is shown in Figure 59. SDS-polyacrylamide gel electrophoresis of the fractions obtained from denatured-DNA cellulose showed that the enzymes were not pure. However, DNA polymerase I was free of the endonuclease which was found to copurify with it in 5.3.6.3, when assays using supercoiled pJDB219 for 20 minutes at 37°C were analysed by agarose gel electrophoresis. The presence of a small amount of contaminating activity was indicated by the observation that extended incubations (6 hours at 37°C) caused the formation of nicked circular plasmid (data not shown).

The temperature-sensitivity of the separated enzymes was assayed on an activated DNA template (2.14) at 23°C, 30°C and 38°C. The enzymes were compared with Calf Thymus DNA polymerase A2 (a gift from Dr. A.M. Holmes). The results are shown in Table XIV. Neither DNA polymerase I or DNA polymerase II showed temperature-sensitivity, which confirmed the results using crude extracts described in 5.3.3.

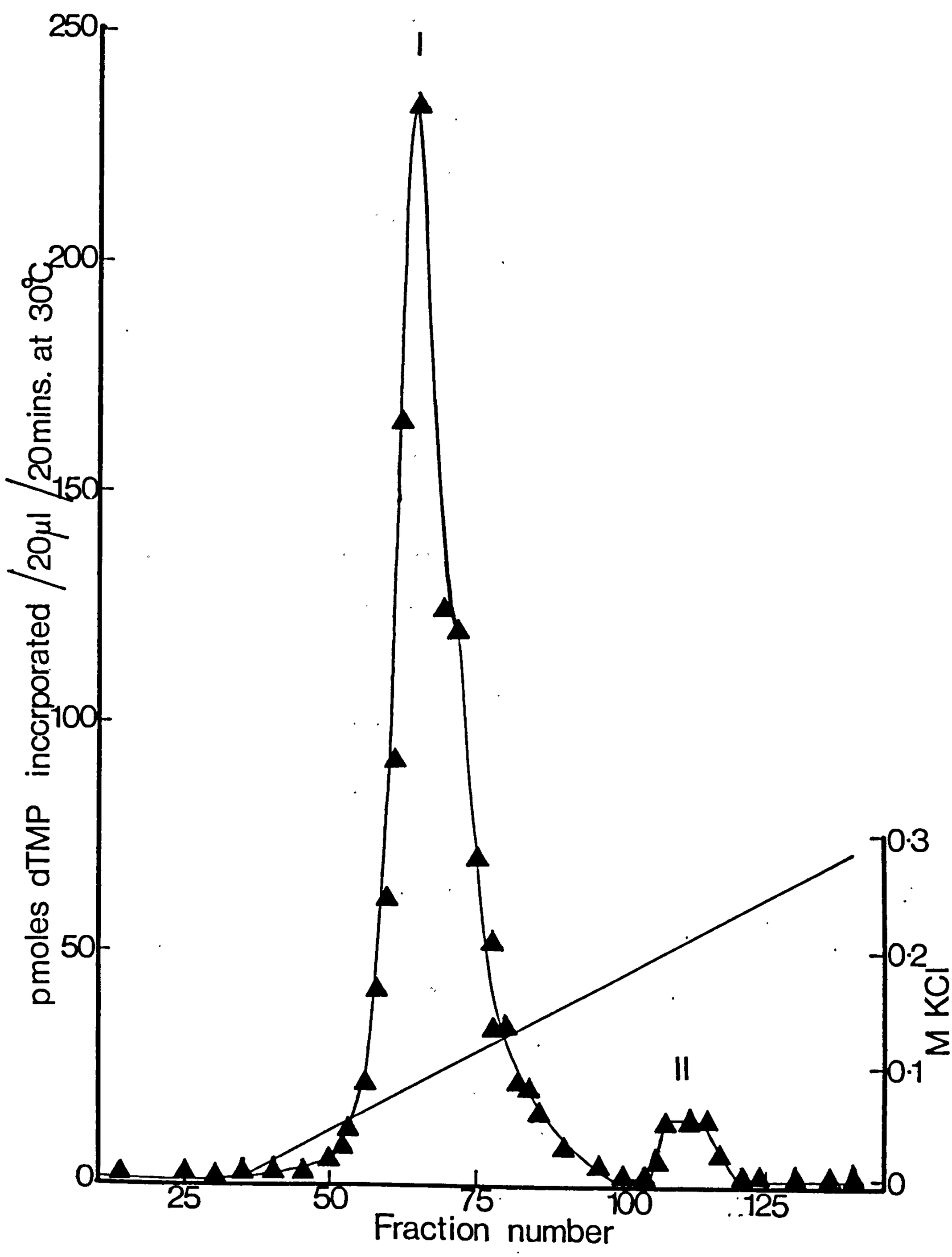
Table XIV. Comparison of DNA polymerase activity at 23°C, 30°C and 38°C.

Enzyme	pmoles dTMP incorporated using activated DNA template.		
	23°C	30°C	38°C
Yeast DNA polymerase I	146	311	549
Yeast DNA polymerase II	15	34	49
Calf Thymus DNA polymerase A2	371	642	951

Assays were performed as described in 2.14 using 20 µl yeast DNA polymerases I and II (27 and 37 µg protein respectively) and 10 µl calf thymus polymerase A2 (7 µg protein).

Figure 59: Separation of yeast DNA polymerases I and II on DEAE-cellulose.

After ammonium sulphate precipitation and dialysis the peak of DNA polymerase activity from phosphocellulose (6.2.3) was loaded onto a 35x2.7cm DEAE-cellulose column equilibrated in 25mM, potassium phosphate, pH7.2; 5mM β-mercaptoethanol, 10% v/v glycerol. The column was eluted with a 1 litre 0-0.5M KCl gradient. DNA polymerase activity was assayed as described in 2.14.


250
200
150
100
50
0
pmoles dTMP incorporated /20µl /20mins. at 30°C
I
II
0·3
0·2
0·1
0
M KCl
25
50
75
100
125
Fraction number

6.3.4.2. Purification of yeast RNA polymerases I, II and III.

RNA polymerases were obtained from commercial yeast strains (2.2) by the method of Valenzuela et al (1978) as described in 6.2.4. The final specific activities of RNA polymerases I, II and III were 4.1, 55.4 and 22.3 units/mg respectively. Stability of the enzymes during purification was not a major problem, though the inability to remove some ammonium sulphate precipitates by centrifugation necessitated filtration through Whatman GF-C with some attendant loss of activity. However, the enzymes were very unstable on storage at -32°C. Polymerase II was the most stable, retaining 80% of its activity after 6 months at -32°C. Polymerases I and III both lost activity rapidly at -32°C and polymerase III was unstable even at -70°C.

The RNA polymerase preparations were characterised by three criteria: i) salt optimum, ii) band pattern on SDS-polyacrylamide gels and iii) inhibition by α-amanitin. For stability reasons, not all the polymerases were available for characterisation by all of these techniques simultaneously. The polymerases also showed marked differences in their ability to use supercoiled plasmid DNA as template (6.3.4.5).

The salt optima of RNA polymerases I and II is shown in Figure 60. The values were 75mM and 150mM KCl respectively. Differences from the published data (100mM and 250mM KCl, Valenzuela et al, 1978) were probably due to differences in template/protein ratio, since the template concentration (20 μg/assay) was not saturating. However, the lower salt optimum for polymerase I suggests the designations were correct.

The band patterns on SDS-polyacrylamide gels suggested that each of the fractions contained additional proteins besides those attributable to RNA polymerase subunits (data not shown). For RNA polymerase I, bands were visible at 186K, 135K, 48K, 44K, 41K, 36K and 24K which corresponded to those reported by Valenzuela et al, (1978). Additionally, bands were visible at 154K, 62K, 58K, 57.5K, 53K and 51K MW which were not reported. (However, in a later preparation these bands were absent).

Figure 60: Salt optima of RNA polymerase I and II preparations.

RNA polymerases I and II were prepared as described in 6.2.4. Their activity at various KCl concentrations was measured in the assay described in 2.13.2, using 9.8 μg protein for RNA polymerase I (●) and 3.8 μg protein for RNA polymerase II (O).

Figure 61: α-amanitin sensitivity of RNA polymerase II and III preparations.

60 μl assays as described in 2.13.2 were performed using 3.8 μg protein from RNA polymerase II (O) and RNA polymerase III (▲) preparations. A stock solution of α-amanitin (Boehringer) at 6mg/ml in sterile water was diluted into duplicate assays to final concentrations of 20 μg/ml, 200 μg/ml and 2mg/ml.

Figure 60

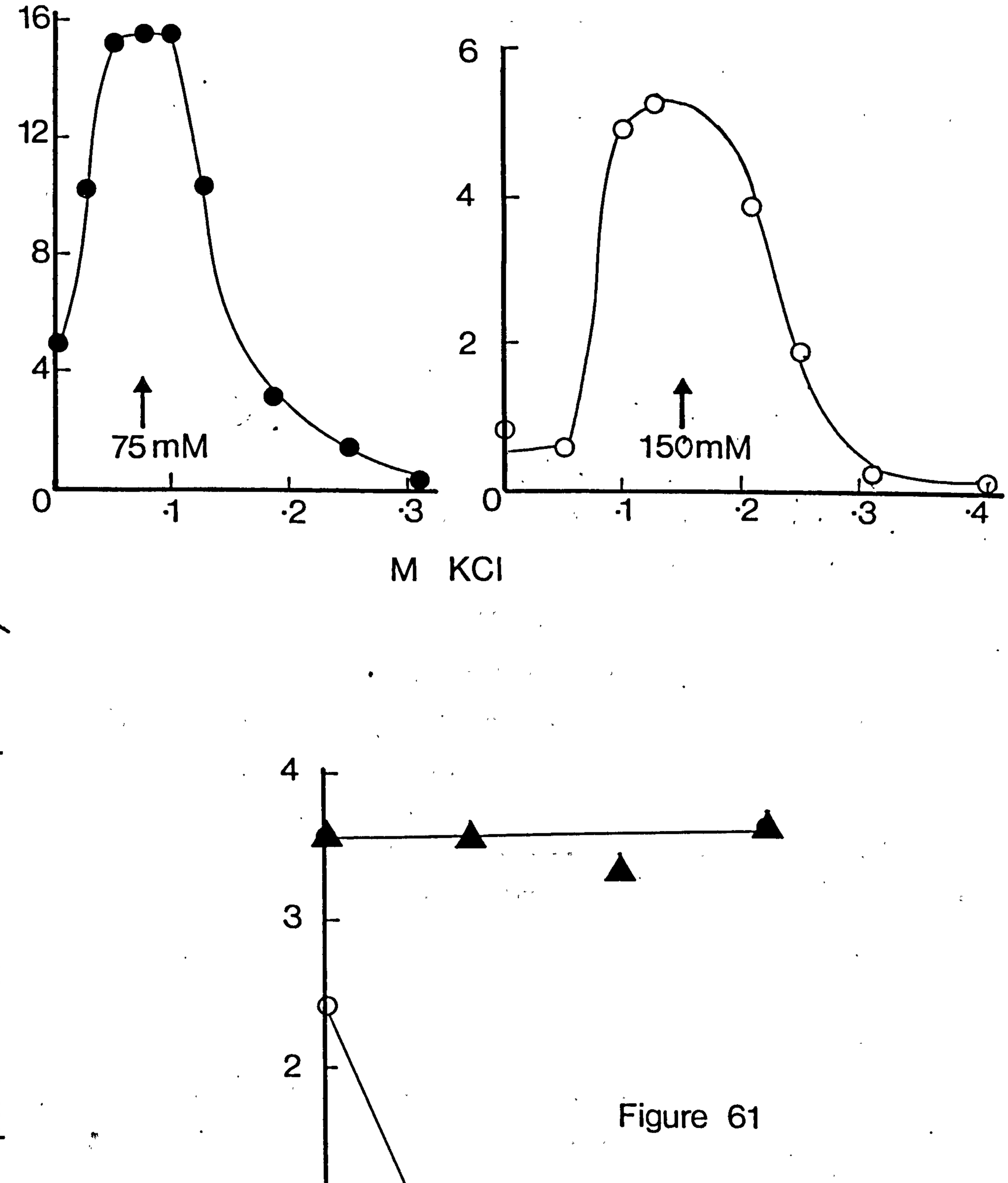


Figure 61

For polymerase II, corresponding bands were seen at 200K, 145K, 46K and 24K, and two bands 35.5K and 28K were absent. Polymerase III showed bands at 160K, 130K, 41K, 37K, 34K, 28K and 20K. A reported band at 82K was not seen. In all cases the lower molecular weight subunits below 28K MW, could not be confirmed on 10% gels.

The α-amanitin sensitivities of polymerases II and III are shown in Figure 61. The sensitivity of RNA polymerase II to 20 μg/ml inhibitor, and the resistance of polymerase III to 2mg/ml were both observed, suggesting that these fractions were correctly identified.

RNA polymerases I and II and DNA polymerase I (6.3.4.1) showed no endonuclease activity as measured by the appearance of open circular DNA on agarose gels (c.f. Fig 49, 5.3.6.3) when assayed for short incubation times (20 minutes at 37°C). Long incubations (6 hours) caused extensive degradation, incidating the presence of contaminating endonuclease activity (data not shown). However, since the assay periods were usually short, this effect was not significant.

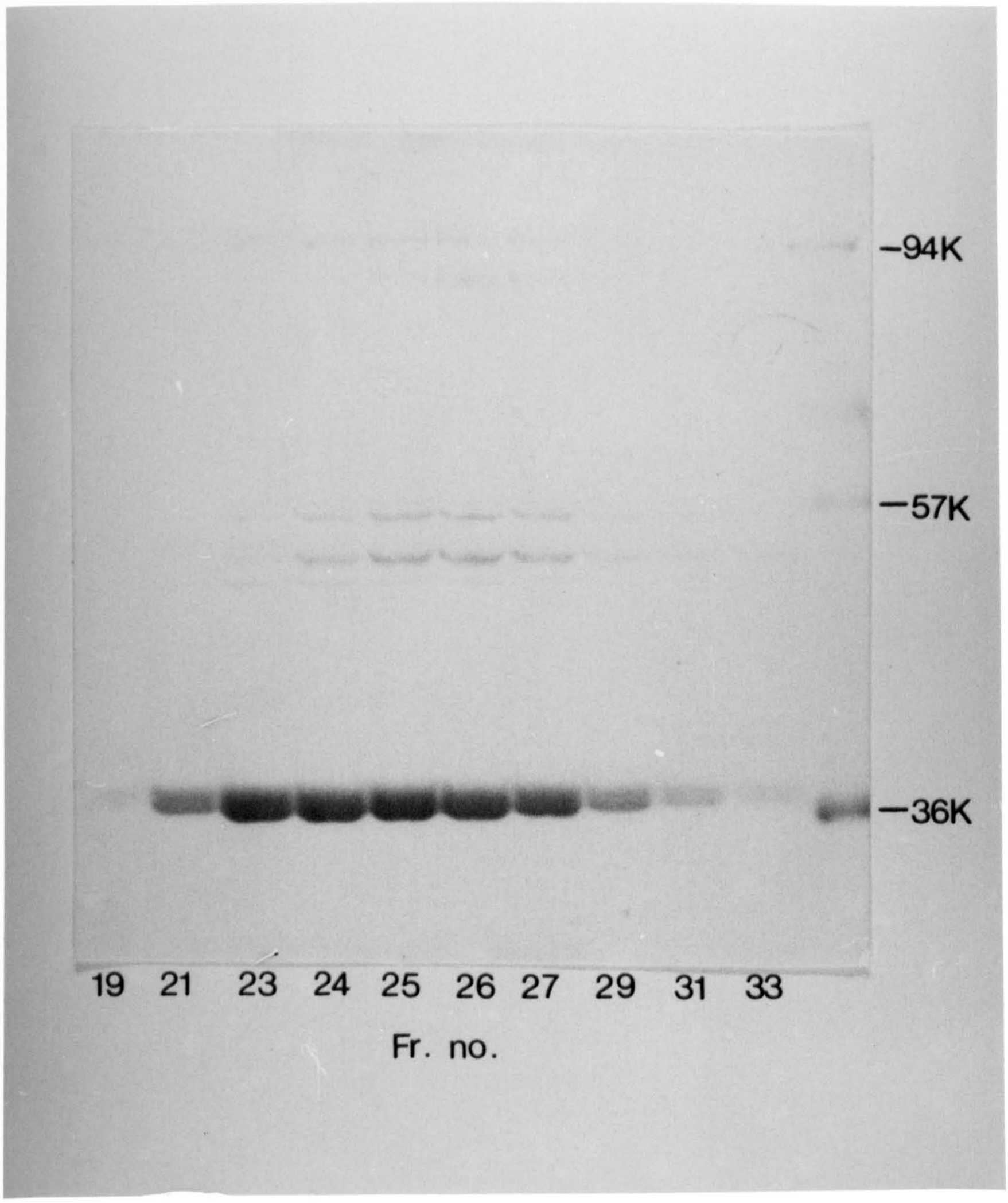
6.3.4.3. Purification of a single strand-specific DNA binding protein.

A crude extract (2.12) was passed through coupled columns of native DNA-cellulose and denatured DNA-cellulose. A symmetrical protein peak was eluted from the denatured DNA-ceelulose, centred at 0.2M NaCl (fraction 25). Samples from the surrounding fractions were electrophoresed on a 10% SDS-polyacrylamide gel. The result, Figure 62, shows that a 37K MW protein band was the major protein species in this peak.

The effect of this protein on DNA replication was studied using a defined synthetic template. The template chosen was $d(pA)_{400}.d(pT)_{10}$, in a 2.5:1 A:T base ratio, the concentration of $d(pA)_{400}$ being 0.1mM nucleotide in the assays. Template and initiator oligonucleotides were hybridised by heating to 55°C for 10 minutes, in 50mM NaCl, 5mM $MnCl_2$, 10mM Tris-HCl pH7.5, then allowing to cool slowly to room temperature (Wickremasinghe, 1975). The effect of various concentrations of the peak

Figure 62: SDS-polyacrylamide gel electrophoresis of single-strand specific DNA binding proteins.

40 µl samples of fractions from the single-stranded DNA-cellulose column described in 6.2.5 were mixed with 10 µl 0.5M Tris-HCl, pH6.8 and 10 µl 10% SDS, heated in a boiling water bath for 5 minutes and electrophoresed on a 10% SDS-polyacrylamide gel as described in 2.10.


—94K
—57K
—36K
19 21 23 24 25 26 27 29 31 33
Fr. no.

fraction 25 (Figure 63) upon DNA synthesis by yeast DNA polymerase I (6.2.3) and calf thymus DNA polymerase A2 (a gift from Dr. A.M. Holmes) was studied at 27°C on the synthetic template and the results are shown in Figure 63. The activity in fraction 25 clearly stimulated both polymerases on this template. The stimulation had not reached a maximum with the largest amount of DNA binding protein added (2.8 μg), and it also appeared to be cooperative. The yeast DNA polymerase I was found to differ from calf thymus polymerase A2 in its template utilisation (Table XV).

Table XV. Template utilisation by yeast DNA polymerase I and calf-thymus DNA polymerase A2.

Enzyme	pmoles dTMP incorporated in 30 minutes at 27°C	
	Activated DNA template (pH7.8)	$d(pA)_{400}.d(pT)_{10}$ (pH7.8)
Yeast DNA polymerase I (27 μg protein)	105	16
Calf Thymus polymerase A2 (7 μg protein)	3409	3.5

Table XVI displays a further investigation of the stimulatory effect of fraction 25. The stimulation was not affected by heating at 80°C for 10 minutes. In fact a slight stimulation was observed. Single-strand DNA binding protein (ssDBP) had no DNA polymerase activity itself. The stimulation did not appear to be due to the addition of nucleic acid in the ssDBP fraction because the observed synthesis was directed by the d(pA).d(pT) template primer, since substitution of ^{3}H-dTTP by ^{3}H-dGTP resulted in no detectable synthesis.

Taking advantage of the heat insensitivity of the ssDBP fraction, an experiment was performed to establish that the activity was due to a protein, because the OD_{280}/OD_{260} ratio for fraction 24 was 1.14, indicating the presence of 2% nucleic acid (Layne, 1957). Table XVII shows the

Figure 63: Effect of single-stranded DNA binding protein (ssDBP) upon DNA synthesis by yeast DNA polymerase I and calf thymus DNA polymerase A2 using a $d(pA)_{400}$• $d(pT)_{10}$ template.

DNA synthesis was monitored in duplicate 50 µl assays containing 50mM Tris-HCl, pH8.0; 200 µM dTTP 11 µCi/ml (methyl-^{3}H) dTTP (31cpm/pmole); 2.5mM $MgCl_2$; 2mM DTT; 10mM NaCl; 1mM $MnCl_2$. The template primer was $d(pA)_{400}$• $d(pT)_{10}$ in a 2.5:1 (A:T) base ratio, with 0.1mM template present (as nucleotide). Assays used 27 µg yeast DNA polymerase I (●) and 7 µg calf thymus DNA polymerase A2 (■) with various amounts of ssDBP (0-2.8 µg). After reaction at 27°C for 30 minutes assays were processed for acid precipitable radioactivity as described in 2.13.

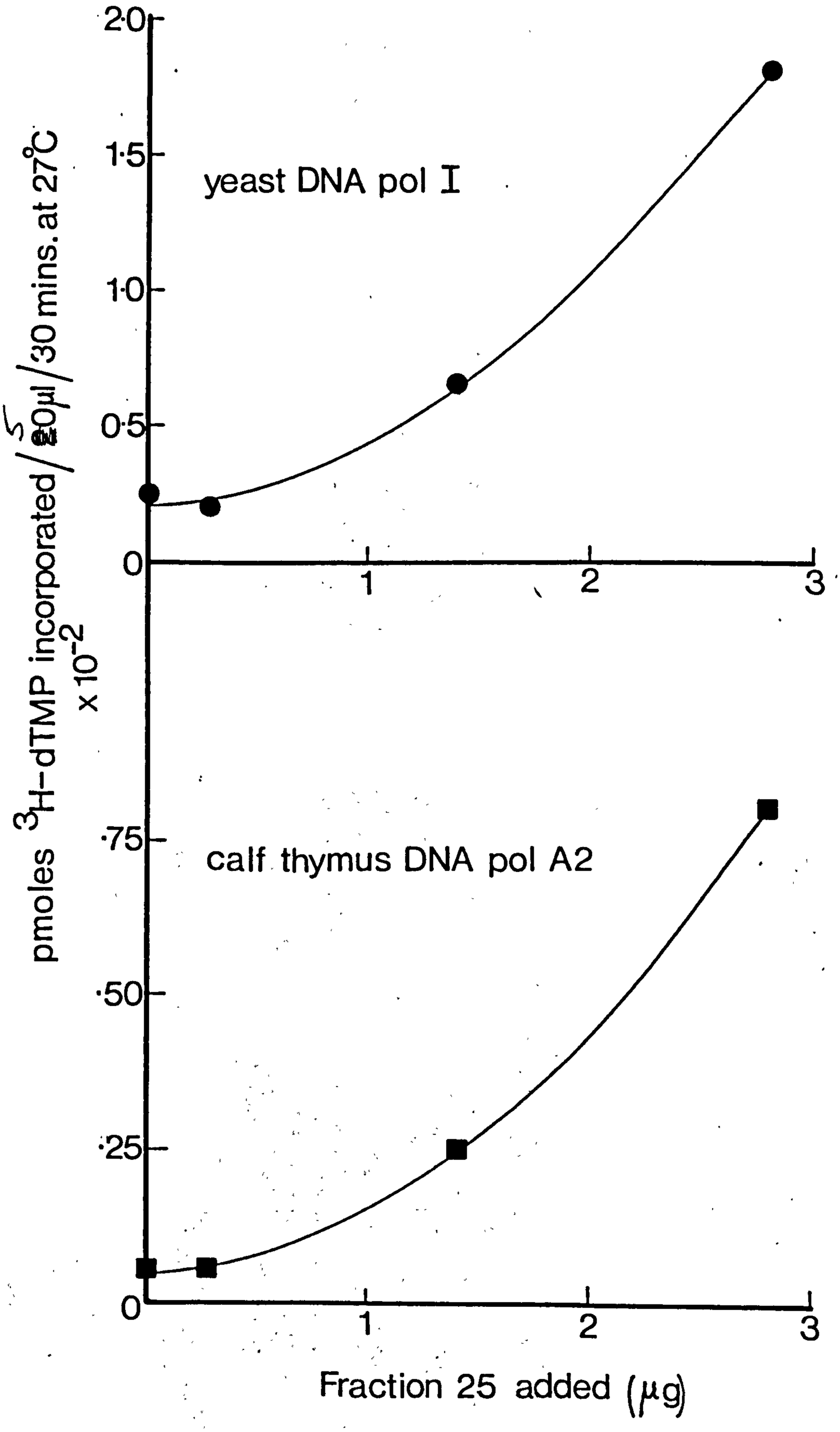

yeast DNA pol I
calf thymus DNA pol A2
pmoles ^{3}H-dTMP incorporated / 20µl / 30 mins. at 27°C x 10^{-2}
2·0
1·5
1·0
0·5
0
·75
·50
·25
0
1
2
3
Fraction 25 added (µg)

Table XVI. Stimulation of DNA synthesis by a single strand-specific DNA binding protein.

Additions	pmoles ^{3}H-dTMP incorporated at 27°C in 30 minutes using $d(pA)_{400}.d(pT)_{10}$ template.
0.2 units DNA polymerase I.	8.6
0.2 units DNA polymerase I + 1.4 µg ssDBP	71.7
0.2 units DNA polymerase I (heated at 80°C for 10 minutes)	0
0.2 units DNA polymerase I + 1.4 µg ssDBP (heated at 80°C for 10 minutes)	89.0
0.2 units DNA polymerase I + 1.4 µg ssDBP + 20mM EDTA	0
0.2 units DNA polymerase I + 1.4 µg ssDBP (^{3}H-dTTP replaced by ^{3}H-dGTP)	0

Table XVII. Effect of carboxypeptidase A, pronase and phosphodiesterase on ssDBP activity.

Yeast DNA polymerase I	ssDBP Treatment	pmoles ^{3}H-dTMP incorporated in 30 minutes at 27°C using $d(pA)_{400}.d(pT)_{10}$ template.
0.2 units	No ssDBP added	8.6
"	1.4 µg ssDBP, incubated 37°C 1 hour then 80°C for 10 minutes	27.2
"	1.4 µg ssDBP + 1mg/ml carboxypeptidase A, incubated 1 hour at 37°C, then 80°C for 10 minutes	7.0
"	1.4 µg ssDBP + 1mg/ml pronase incubated 1 hour at 37°C then 80°C for 10 minutes	16.4
"	1.4 µg ssDBP + 1mg/ml phosphodiesterase + 6mM Mg^{2+} incubated at 37°C for 1 hour, then 80°C for 10 minutes.	24.7

Note: mix specific activity in Tables XVI and XVII was 31 cpm/pmole.

effect of treatment of ssDBP with 1mg/ml carboxypeptidase A, pronase, and snake venom phosphodiesterase (Sigma) at 37°C for 1 hour followed by 80°C for 10 minutes to inactivate the added enzymes. The remaining stimulatory activity was considerably reduced even in a control ssDBP sample incubated without additions. However, the proteases carboxypeptidase A and pronase caused reduction of the ssDBP stimulatory activity whereas phosphodiesterase caused little. Therefore, the stimulatory activity appeared to be due to a protein. The protein also stimulated yeast DNA polymerase I activity on activated DNA (2.14) by 28% in 60 minute assays at 23°C, (data not shown).

6.3.4.4. Attempts to initiate DNA synthesis on supercoiled pJDB219 DNA using purified components.

Using the purified components obtained at this point, namely DNA polymerase I, RNA polymerases I, II and III, and a single strand-specific DNA binding protein, DNA synthesis was assayed on pJDB219 in the reaction mix described in 6.2.7. Assays were performed at 23°C for 60 minutes with the components alone, or mixed in varying concentrations. In no case was significant synthesis observed. Agarose gel electrophoresis (2.17) of phenol extracted samples confirmed that supercoiled plasmid DNA remained at the end of these incubations. Also, 5 µl samples of fractions from a DEAE-cellulose gradient (0-0.5M NaCl) of a crude extract (2.12) were added to 50 µl assays containing 15 µl DNA polymerase I, 3 µl RNA polymerase I, 5 µl RNA polymerase II and 8 µl ssDBP. Again no synthesis was observed. Also a gradient fractionation of native DNA cellulose fractions gave the same result.

6.3.4.5. RNA synthesis in vitro.

Following these experiments two questions needed to be answered. Could the RNA polymerases utilise the supercoiled plasmid as a template and could the DNA polymerase utilise RNA primers synthesised by RNA polymerase? The latter question had been positively answered by Plevani

and Ghang (1977) using single stranded DNA. The former question represented an entire field of research in itself.

The utilisation of pJDB219 DNA as a template by the RNA polymerases was studied using an RNA polymerase reaction mix as described in 2.13, the template being 20 μg/ml pJDB219 DNA in the assay. Various amounts of RNA polymerases I, II and III were tested in this mix at 30°C for 30 minutes. The results for RNA polymerases II and III are shown in Figure 64. Two preparations of RNA polymerase I failed to transcribe supercoiled DNA. Figure 64, however, shows that an optimum concentration of RNA polymerase II at a ratio of 3.9 μg protein/1.3 μg plasmid DNA was found. Much more extensive synthesis was seen with RNA polymerase III. It was therefore of interest to discover whether the observed transcription was occurring at specific sites on the plasmid DNA, or whether it was distributed at random. pJDB219 has 4 EcoR1 sites (Figure 65). Several samples of EcoR1 cut and uncut plasmid DNA were separated in adjacent tracks on a 1.4% agarose gel (2.17), as shown in Figure 65. The DNA was denatured and transferred to a nitrocellulose filter as described in 6.2.6. ^{32}P-labelled RNA transcripts were prepared using RNA polymerases II and III in RNA polymerase reactions mixtures as used in Figure 64, but of final volume 0.35ml, with ^{3}H-rUTP replaced by (α-^{32}P) rUTP. Samples were removed from these incubations at 5 minutes and 20 minutes into the reaction. Preliminary observations had shown that the rate of transcription by RNA polymerase II was linear for up to 20 minutes; RNA polymerase III transcription began to decline after 10 minutes (data not shown). Figure 66 shows that RNA polymerase II transcribes selectively band III in Figure 65, with material hybridising to band II after 20 minutes incubation. RNA polymerase III transcribes specifically the DNA in bands I and II.

From an inspection of the map of pJDB219 (see legend to Figure 65) these results indicate that RNA polymerase II transcribes specifically the region of the plasmid encoding the yeast leu 2 gene, and RNA

Figure 64: Transcription of pJDB219 DNA by yeast RNA polymerases II and III.

RNA synthesis using pJDB219 as template was measured in the assay described in 2.13.2, with calf thymus DNA replaced by 20 μg/ml pJDB219, and 15mM KCl present. Assays used 0-9.8 μg RNA polymerase II (O) and 0-21.0 μg RNA polymerase III (▲). The specific activity of (methyl-^{3}H) rUTP was increased to 1050 cpm/pmole in this assay, and reactions were performed at 30°C for 30 minutes.

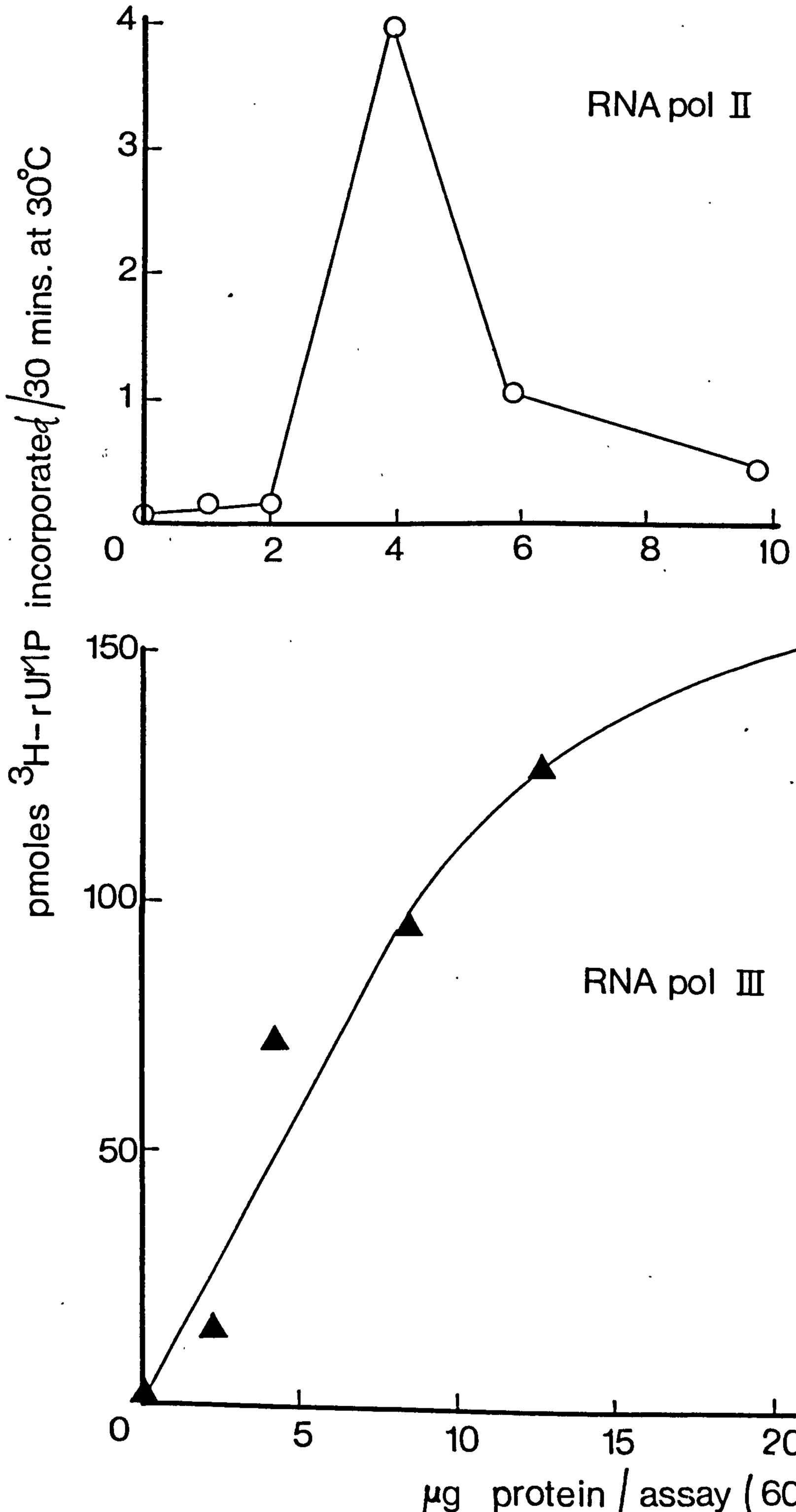
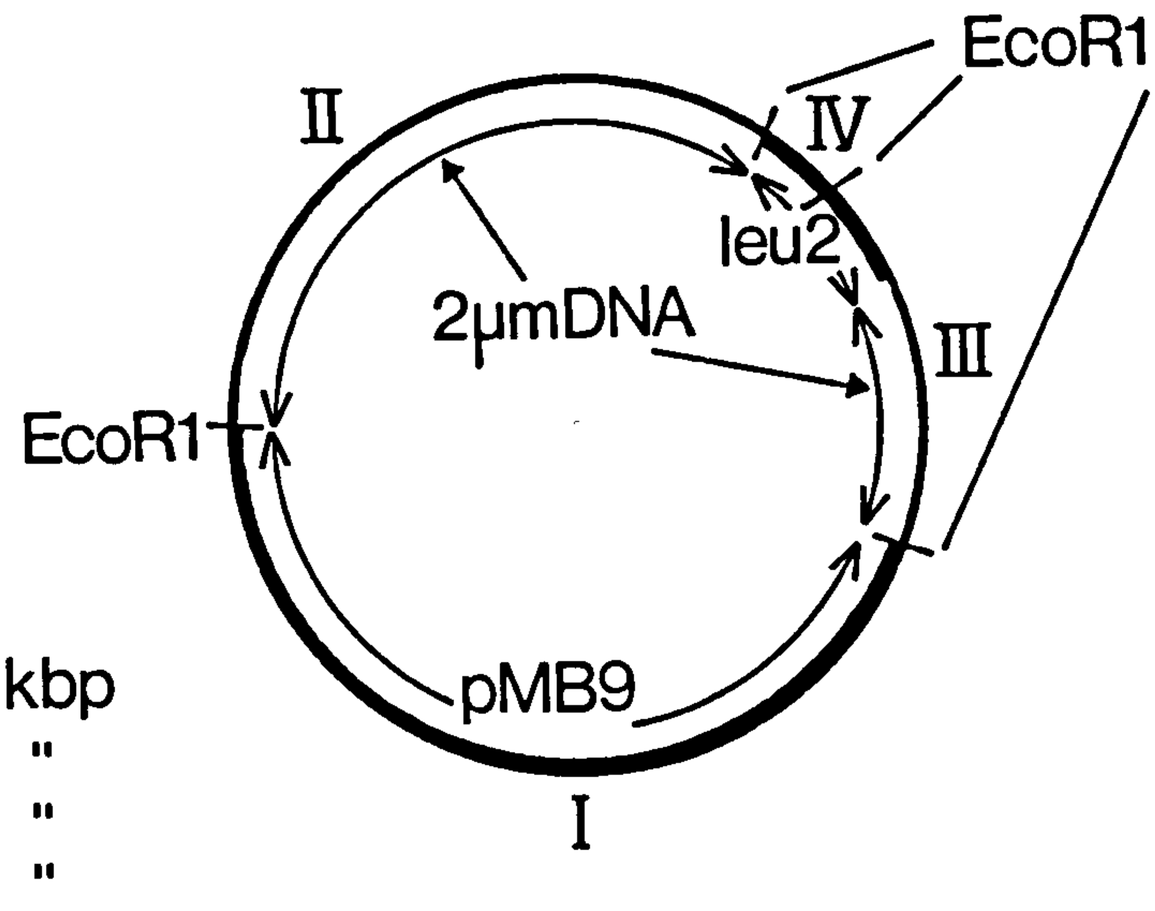

4
3
2
1
0
2
4
6
8
10
pmoles incorporated /30 mins. at 30°C
RNA pol II
150
100
50
0
5
10
15
20
25
pmoles ^{3}H-rUMP
RNA pol III
µg protein / assay (60µl)

Figure 65: Treatment of pJDB219 with EcoR1 restriction enzyme.

A 223 µl digest was prepared containing 0.1M Tris-HCl, pH7.5; 50mM NaCl, 10mM $MgCl_2$, 26 µg pJDB219, DNA and 30 µl diluted EcoR1 enzyme (Boehringer; a 1 in 12 dilution of enzyme in 10mM potassium phosphate, pH7.0; 0.2M NaCl, 1mM EDTA, 0.7mM β-mercaptoethanol, 2mg/ml BSA, 0.2% v/v Triton X-100). The digest contained 200 units of EcoR1 activity (Boehringer), and was performed at 37°C for 10 minutes. 75 µl of stop mix (2.17) was added, and six 45 µl portions were run in a 1.4% agarose gel with 3 µg undigested pJDB219 DNA in adjacent tracks. A map of pJDB219, with the identification of bands I, II, III and IV is shown below.



pJDB219.

Figure 66: Hybridisation of transcripts to pJDB219 DNA.

0.35ml incubations were performed using the conditions as in Figure 64 with 39 µg RNA polymerase II and 34 µg RNA polymerase III and 4.2 µCi/ml (α-^{32}P)-rUTP. 170 µl samples were removed at 5 minutes (tracks A and C), and 20 minutes (tracks B and D). These samples were extracted once with redistilled phenol (equilibrated with 0.1M Tris-HCl pH8.0), and once with chloroform. Sodium acetate was added to a final concentration of 0.3M, and the DNA was precipitated in 70% ethanol overnight at -32°C. After dissolving the pellets in 100 µl sterile water, removal of (α-^{32}P)-rUTP was effected by passage of the samples down sterilized 22x0.5cm Sephadex G-50 columns. The DNA in the agarose gel shown in Figure 65 was transferred to nitrocellulose sheet as in 6.2.6. and the RNA polymerase II (A and B) and RNA polymerase III (C and D) transcripts were hybridised as in 6.2.6.

Figure 65

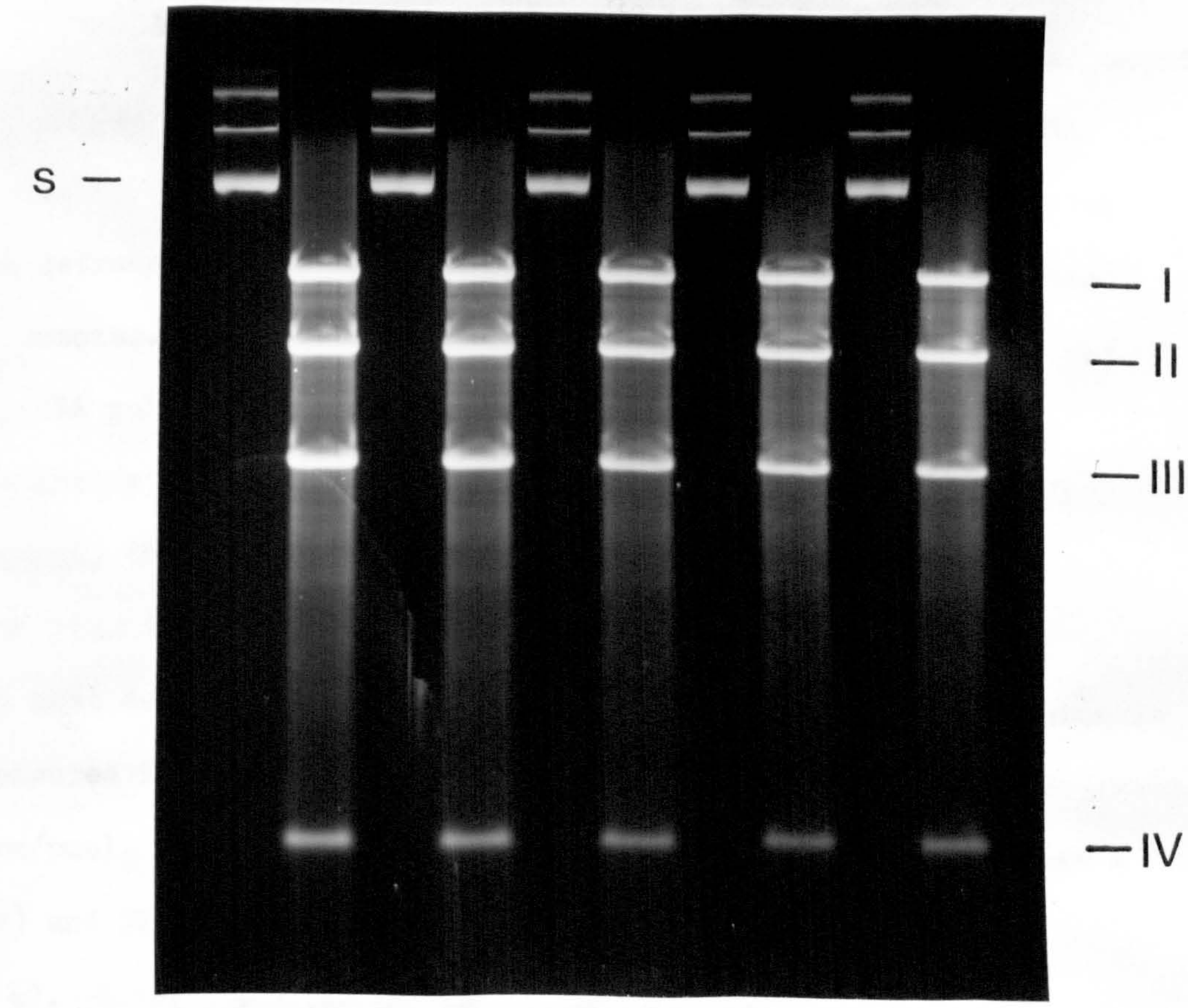
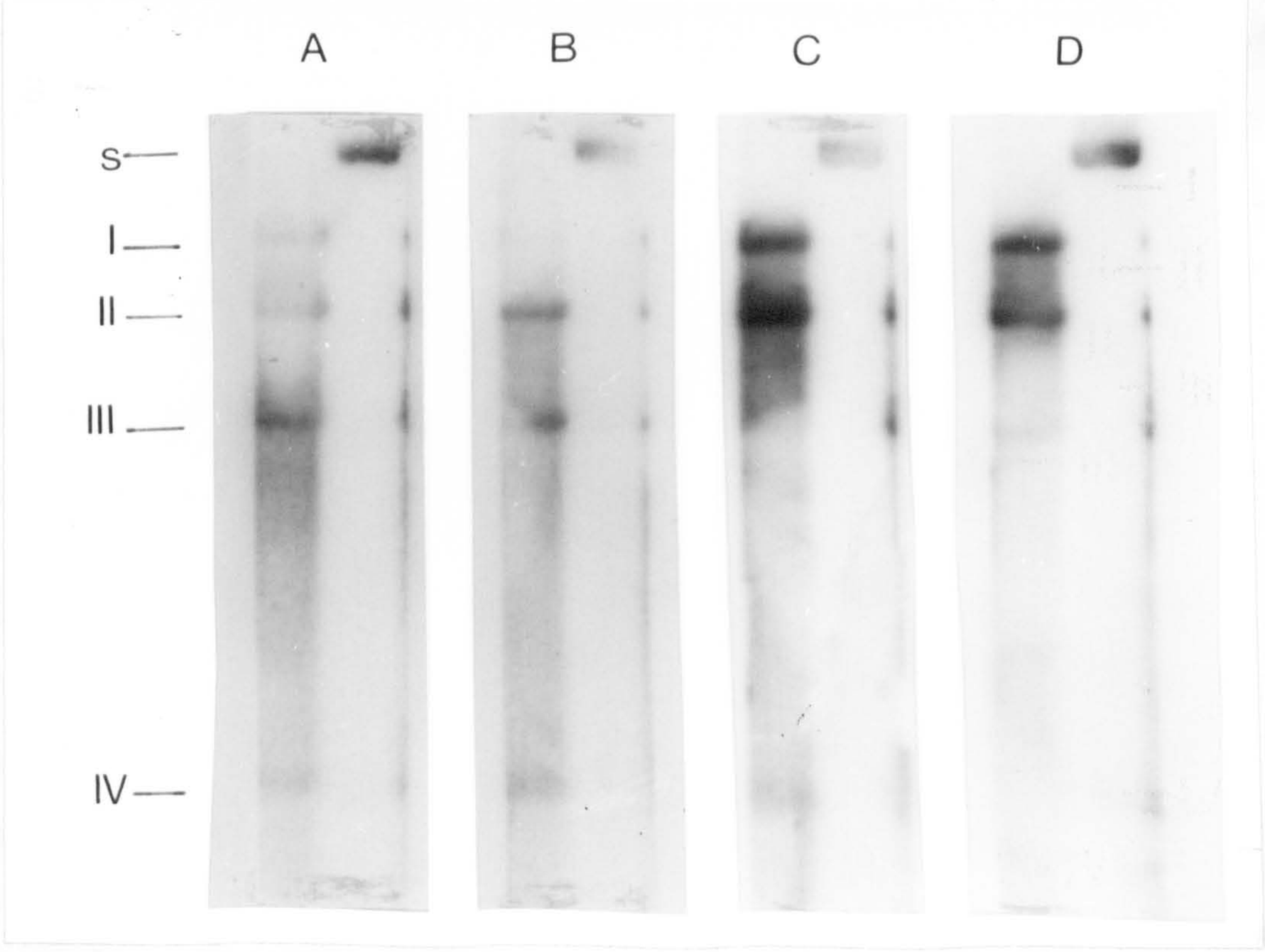


Figure 66



polymerase III transcribes the sequences derived from pMB9 and adjacent yeast 2 μm DNA sequences (Bands I and II in Figure 65). Thus RNA polymerase III appeared to transcribe from the prokaryotic promoter for the tetracycline gene, and RNA polymerase II utilised the yeast leu 2 promoter. In keeping with other observations (Van Keulen and Retel, 1977), RNA polymerase I failed to utilise supercoiled DNA.

Returning to the question of priming of DNA synthesis by RNA polymerases, this was tested in the reaction mix described in 6.2.7, but with plasmid DNA replaced by a final concentration of 80 μg/ml freshly heat denatured calf thymus DNA (Sigma). RNA and DNA synthesis were measured in duplicate mixes using ^{3}H-rUTP and ^{3}H-dTTP respectively (1000cpm/pmole each). The test was performed using RNA polymerase I (9.8 μg) and DNA polymerase I (3.3 μg). The results are shown in Figure 67. Although the observed DNA synthesis was low, a definite stimulation by RNA polymerase was apparent. The results also confirmed that RNA polymerase I had no DNA synthetic ability and conversely DNA polymerase I had no RNA synthetic ability.

Thus DNA polymerase I as used in the assays in 6.3.4.4, had the capacity to utilise RNA primers to synthesise DNA. Also, there was evidence to suggest that RNA polymerases II and III were capable of transcription from various parts of the pJDB219 plasmid (Figures 65 and 66). This suggested that the failure to observe DNA synthesis in reconstituted assays was caused by a lack of essential components in the reaction, and was not due to inactivity of the purified components.

6.3.4.6. Purification of nicking-closing enzyme (DNA topoisomerase).

In section 5.3.6, an activity referred to as "fraction 75" (Figure 44) caused apparent faster migration of supercoiled DNA (Figure 52). Evidence suggested that this was due to a topological alteration of the plasmid (Figure 51). It was realised that a possible solution to the problem of opening the double helix at a replication origin without the

Figure 67: Synthesis of DNA and RNA on single-stranded DNA using DNA polymerase I and RNA polymerase I.

Using the in vitro assay conditions described in 6.2.7, but containing 80 μg/ml heat-denature calf thymus DNA, and 9 μCi/ml of either (methyl-^{3}H)rUTP or (methyl-^{3}H)dTTP, (1000cpm/pmole for both). Assays were performed using 9.8 μg RNA polymerase I (O) or 3.3 μg DNA polymerase I (□) or both together (△). In all cases, 120 μl assays were incubated at 30°C, and 25 μl samples were removed at the times indicated for processing as described in 2.13. Figure 67A shows the results for DNA synthesis (open symbols) and Figure 67B, the results for RNA synthesis (solid symbols).

Figure 67A

DNA synthesis

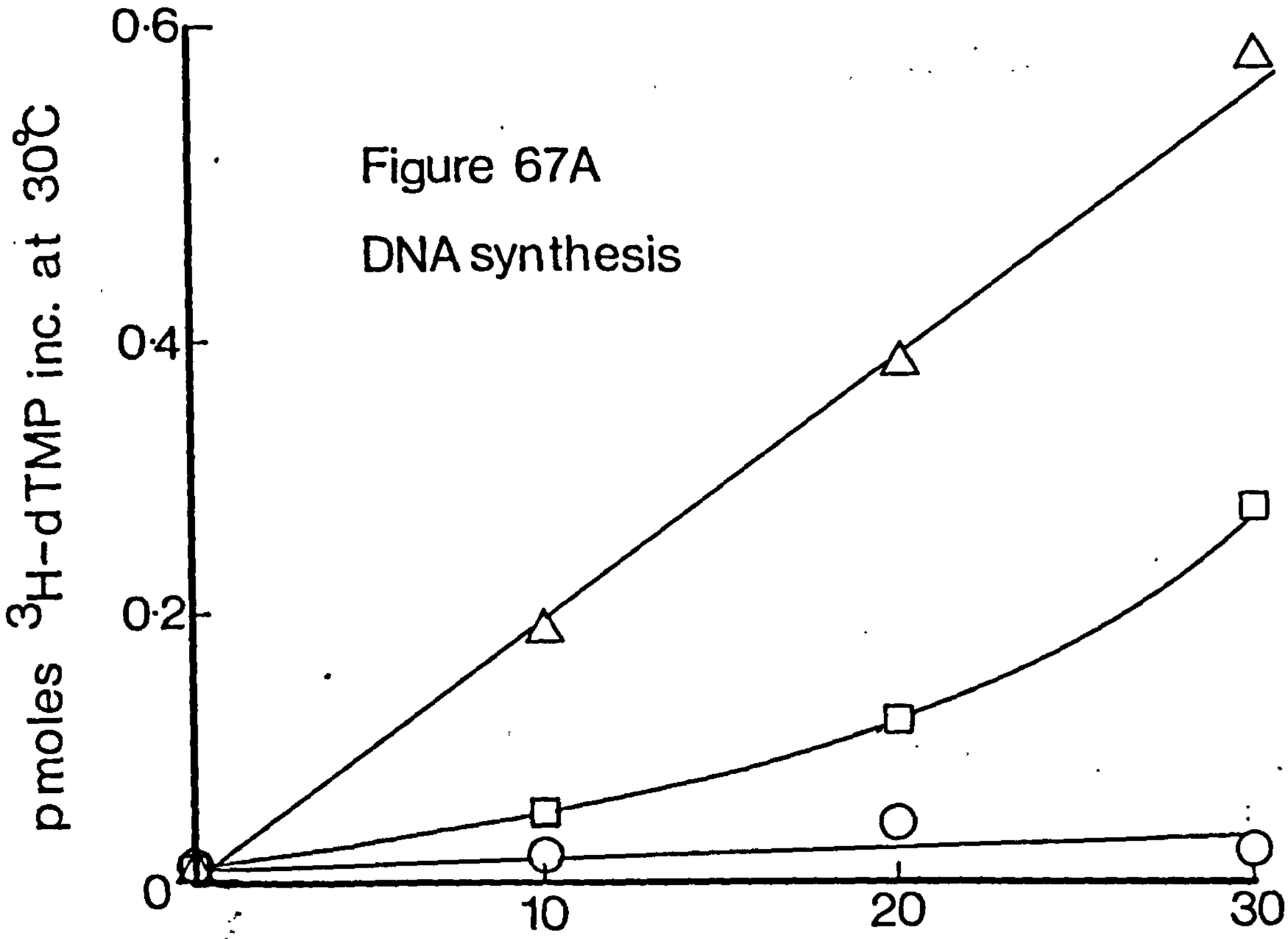
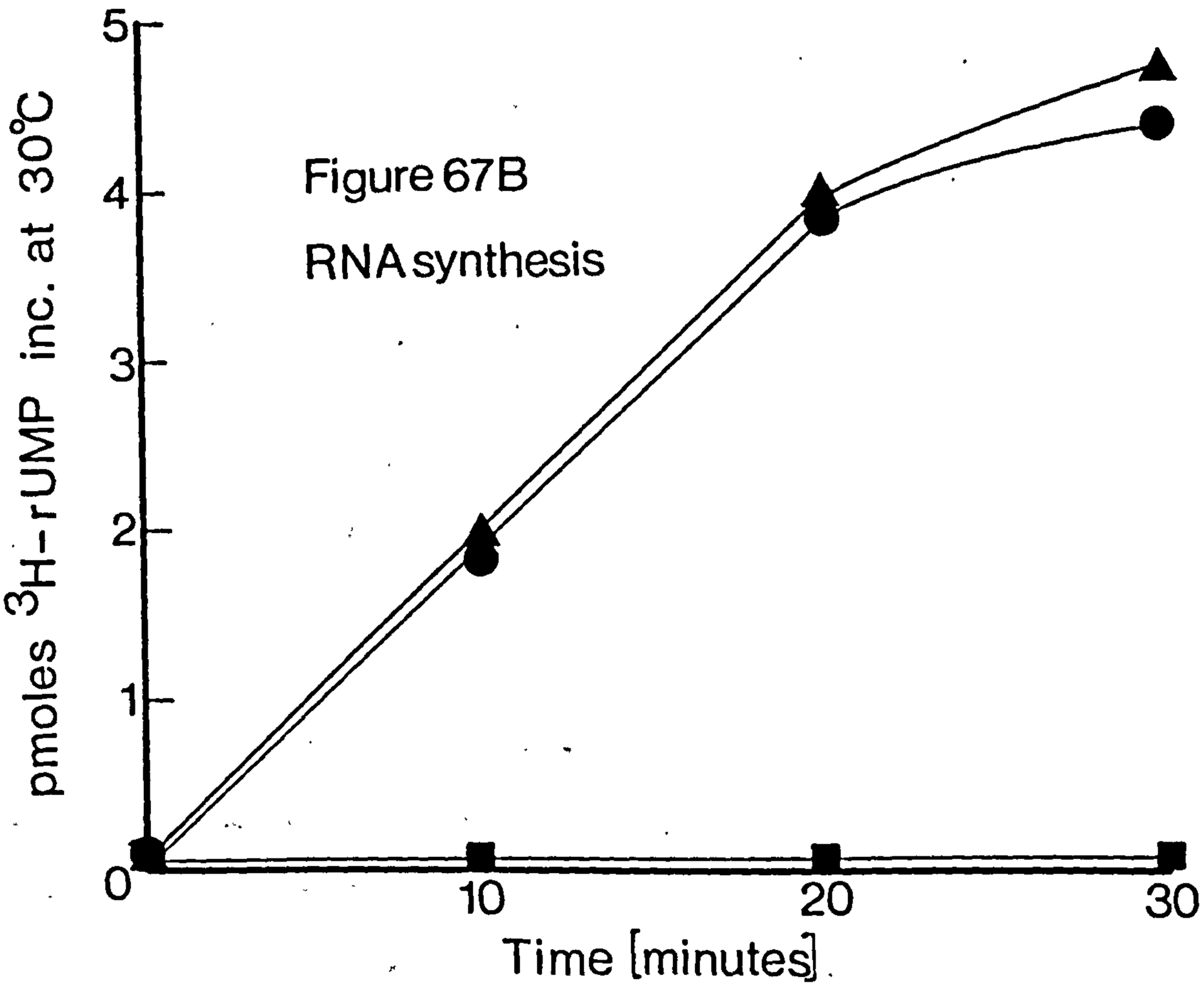


Figure 67B

RNA synthesis



necessity for an endonuclease to produce a 3^{1}OH end, would be underwinding of the superhelix via a DNA gyrase-like activity, causing helix destabilization in a limited domain. Some time later, Liu et al, (1979) suggested just such an activity for the bacteriophage T4 gene 39, 52 and 60 products, mutants of which were known to be defective in the initiation of DNA synthesis. The activity of "fraction 75" was known to be not thermosensitive, but it seemed possible that this activity was an essential component of the initiation/replication machinery.

However, the true nature of this activity was revealed when the DEAE-cellulose fractionation described in 5.3.6 was repeated. It must be noted that the agarose gels depicted in Chapter 5 were electrophoresed according to the legend to Figure 48, the electrophoresis buffer containing 0.5 μg/ml ethidium bromide.

A crude cell extract from cdc7.4 cells prepared as in 5.2.7 was fractionated on DEAE-cellulose DE52, using a 0-1M NaCl gradient. The fractions in the 0.04-0.10M NaCl region of the salt gradient were assayed for 3 minutes at 38°C with 1.5 μg pJDB219 DNA in 50mM Tris-HCl pH8.0, 7mM $MgCl_2$, then the samples were electrophoresed in a 0.8% agarose gel precisely as described in 2.17. The result, Figure 68, showed no fast migrating DNA species; instead, fraction 31 caused the plasmid to run in the position of open circular DNA. When pJDB219 DNA samples treated with this fraction, as in the time course shown in Figure 69, were run in a 0.8% agarose gel containing 0.5 μg/ml ethidium bromide, the fast migrating DNA was seen. Clearly, this result was an artefact of electrophoresis in the presence of ethidium bromide. Electron microscopy of Kleinschmidt-spread, phenol-extracted pJDB219 following reaction with fraction 31, confirmed that the product was relaxed circular DNA (Figure 70). The optimum salt concentration for the formation of relaxed DNA was determined in 30 μl reactions containing 50mM Tris-HCl, pH8.0, 1.5 μg pJDB219 DNA, and added KCl of varying concentrations. The result of agarose gel electrophoresis is shown in

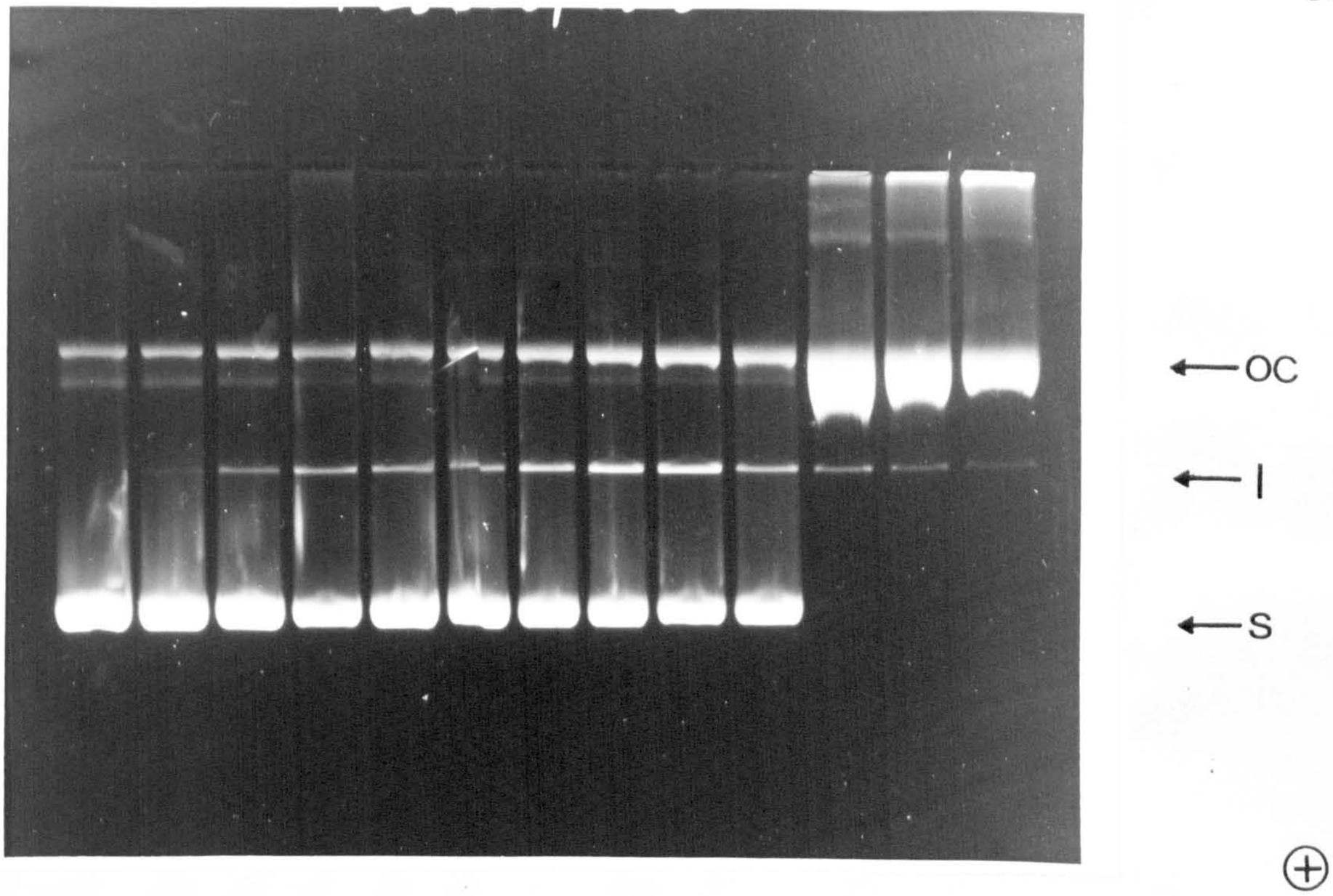
Figure 68: Assay of DEAE-cellulose gradient fractions on an agarose gel.

A crude extract from log-phase cdc7.4 (DE200.1.3) cells was prepared as in 5.2.7 and fractionated on DEAE-cellulose as in Figure 44 except that the column was 39.5x3.0cm, and a 1 litre 0-0.5M NaCl gradient was used. Fractions 21-33 (in the 0.04-0.1M NaCl region of the gradient) were assayed for their effects in pJDB219 DNA in 50mM Tris-HCl pH8.0, 7mM $MgCl_2$ as in Figure 52, and the reaction products were analysed on a 0.8% agarose gel as described in 2.17 (Note, no ethidium bromide was present during the electrophoresis) s, supercoil; l, linear; oc, open circle.

Figure 69: A time-course of the effect of fraction 31 upon pJDB219 using agarose gel electrophoresis in the presence of ethidium bromide.

A 360 µl assay was performed using 108µg fraction 31 protein under the same conditions used in Figure 68. 30µl samples were removed at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 5.0, 7.0, and 15.0 minutes after mixing. Electrophoresis was as described in Figure 52, i.e. in the presence of 0.5µg/ml ethidium bromide. f = fast migrating DNA.

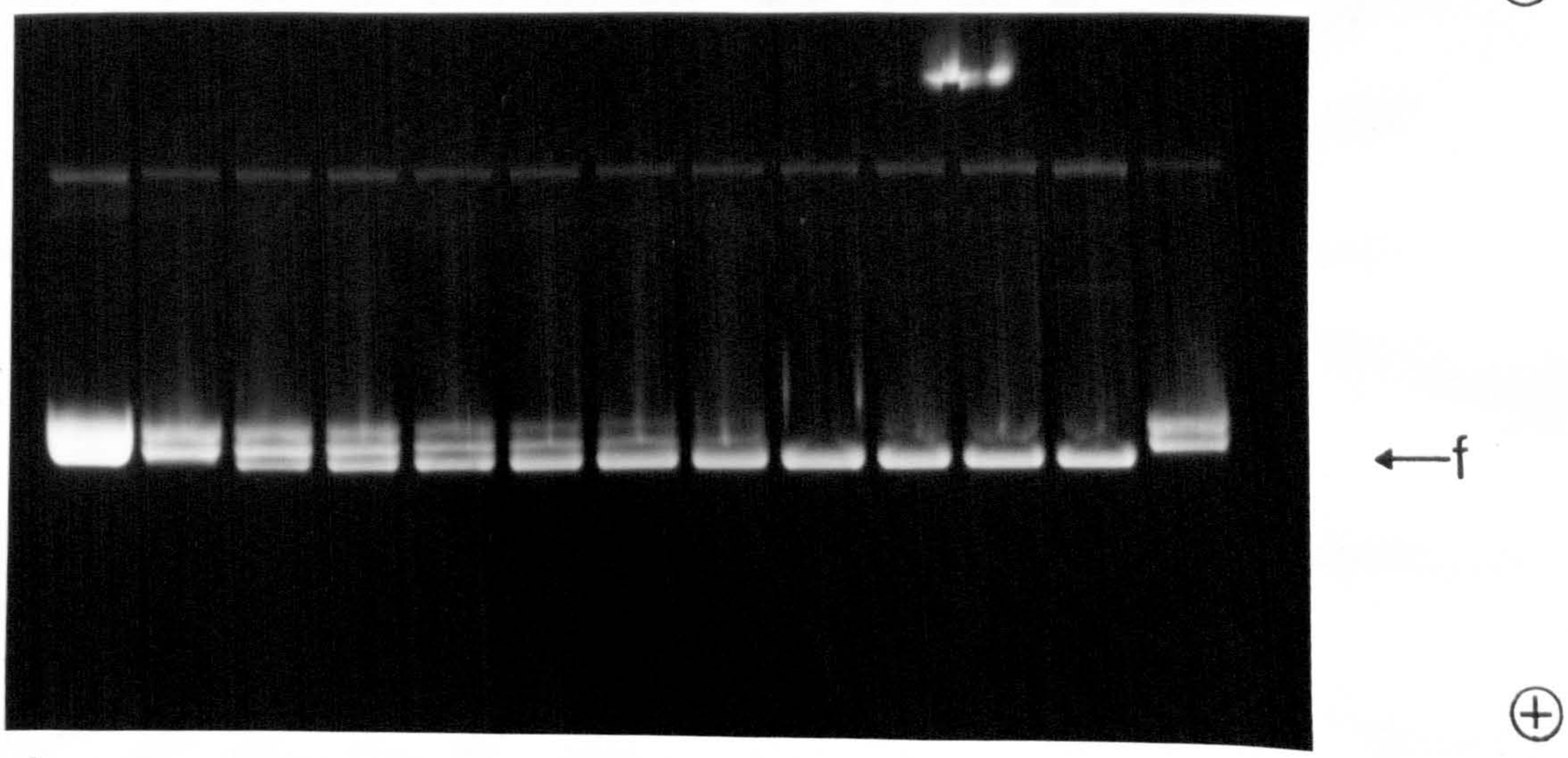
Figure 68



Enz. dil.	22	23	24	25	26	27	28	29	30	31	32	33

Fr. no.

Figure 69



minus enz.	0	0·25	0·5	0·75	1	1·5	2	3	5	7	15

Time mins.

Figure 70: Electron microscopy of pJDB219 DNA before and after reaction with fraction 31.

10 μg pJDB219 DNA was completely converted to fast migrating DNA as in Figure 69 by reaction with 9 μg fraction 31 protein. The DNA was phenol and chloroform extracted, ethanol precipitated and Kleinschmidt spread as in 2.18.

Figure 70A: shows supercoiled pJDB219 not treated with fraction 31.

Figure 70B: shows the product of the reaction with fraction 31.

A

B

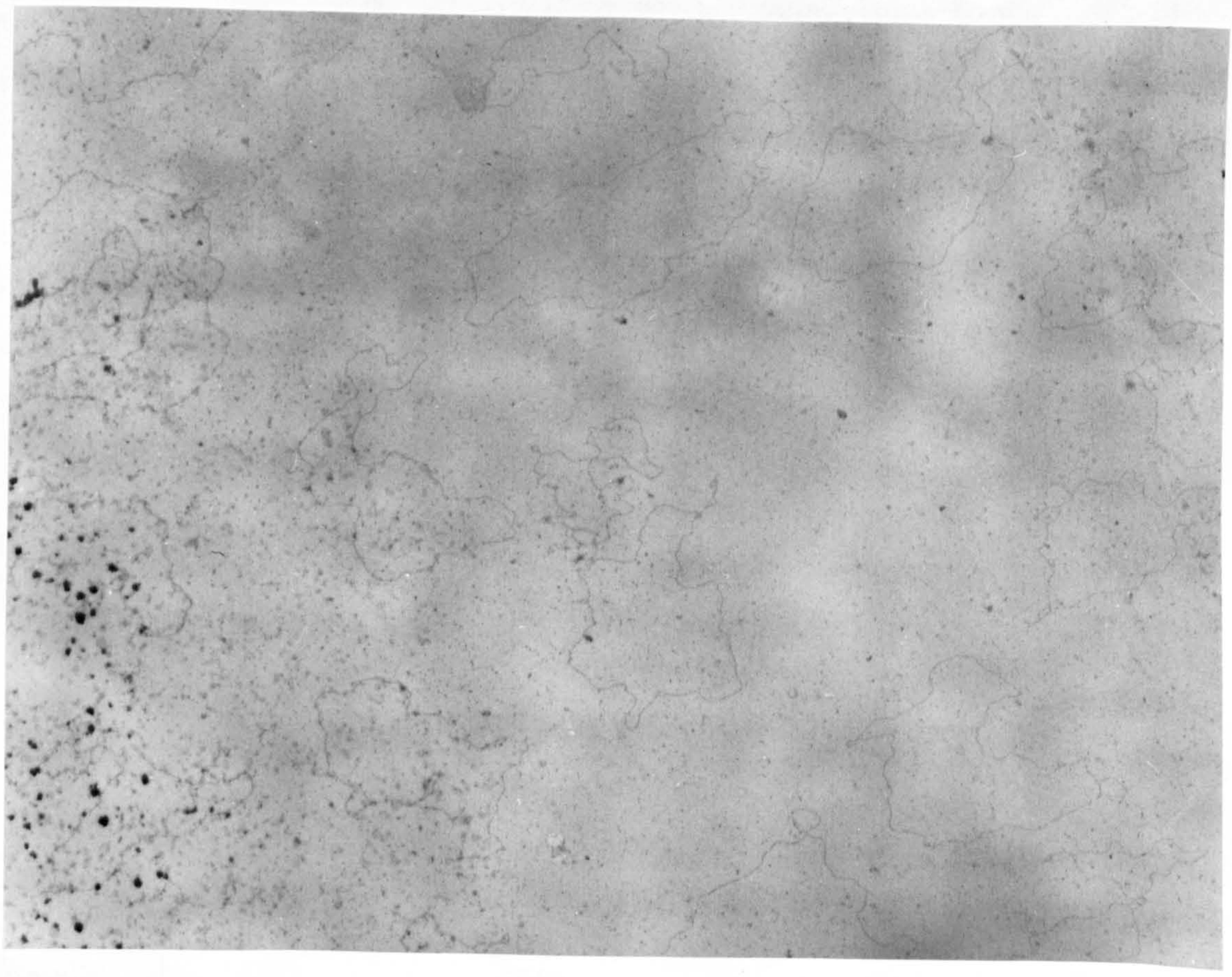


Figure 71: Salt optimum of yeast DNA topoisomerase.

30 µl reactions contained 50mM Tris-HCl pH8.0, 1mM DTT, 1.5 µg pJDB219 DNA, 2.5 µg fraction 31 proteins and 0-0.5M KCl. Reactions were for 3 minutes at 38°C.

Figure 72: Relaxation of positive and negative superhelical turns by yeast DNA topoisomerase.

Reactions were performed as in Figure 71 using 75mM KCl, and containing 0, 1.5, 5 or 10 µg/ml ethidium bromide. Control reactions were performed for each concentration using enzyme diluent (Figure 45). After reactions for 15 minutes at 37°C, enzyme and ethidium bromide were removed by phenol and chloroform extractions and the samples run on a 0.8% agarose gel.

Figure 71

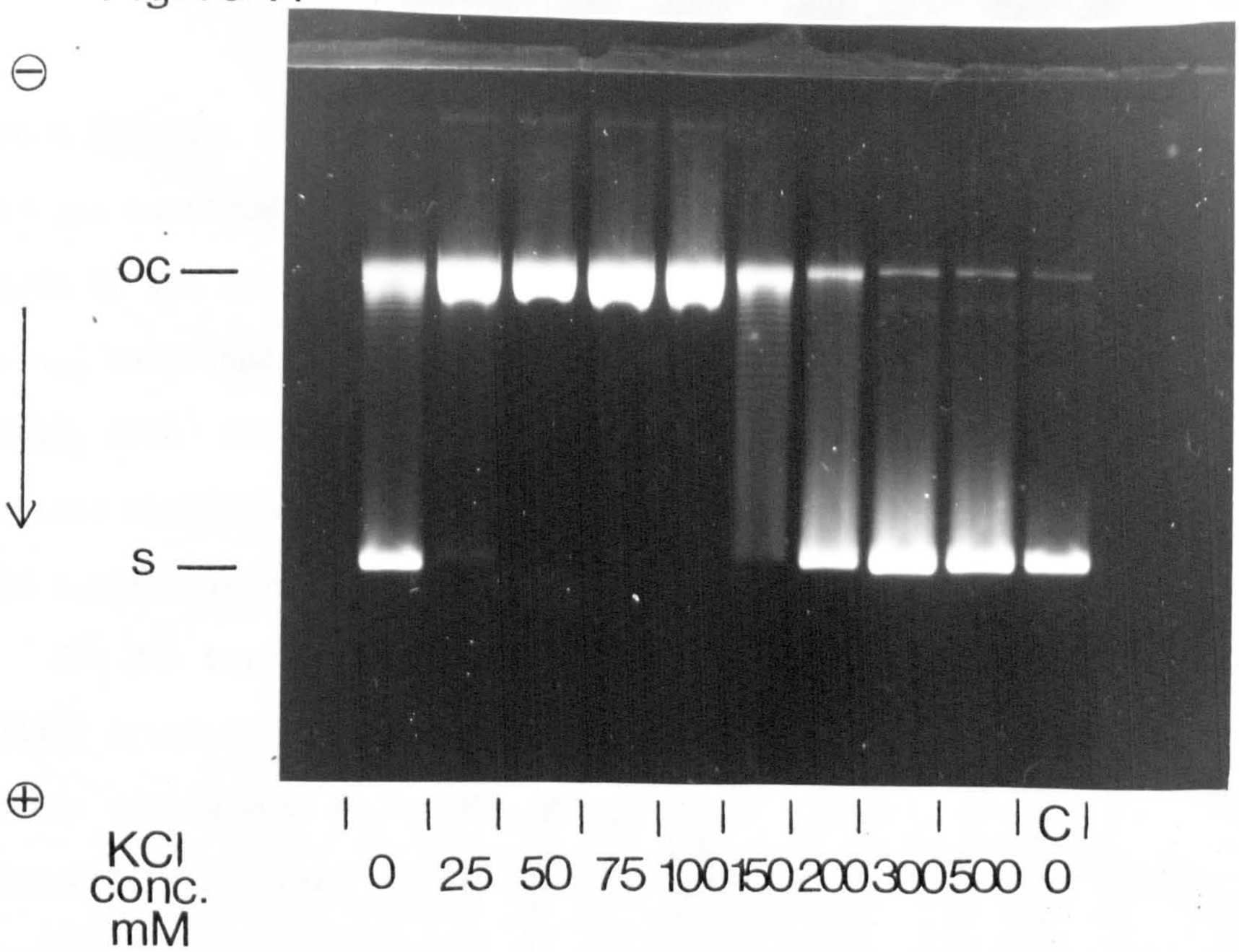


Figure 72

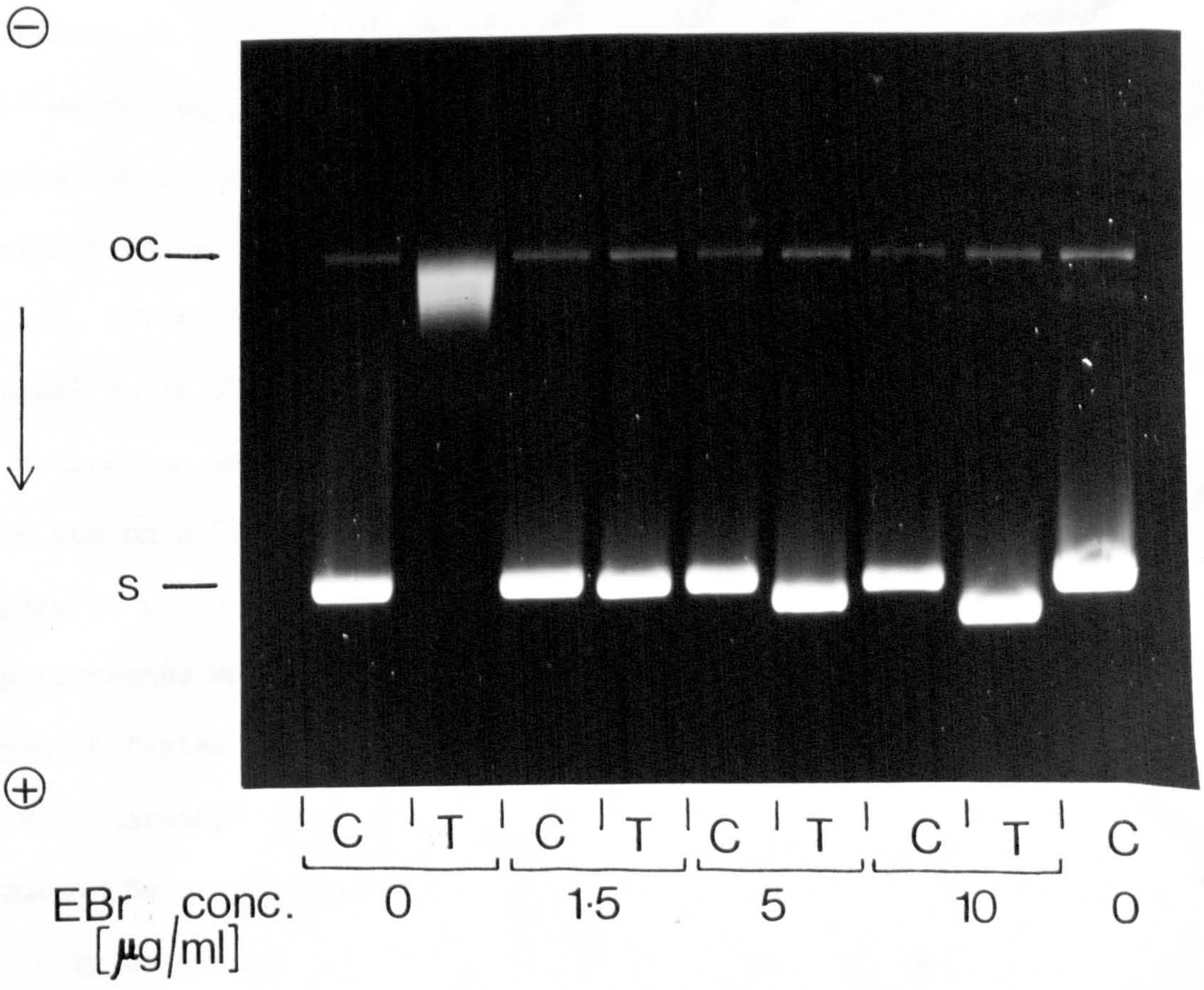


Figure 71. Not only was a salt optimum of 75mM KCl observed, but this gel confirmed that the activity in fraction 31 was a nicking-closing enzyme by the appearance of a ladder of intermediate bands (tracks 1 and 6), each band representing a DNA species whose linking number, L, (Crick, 1976) differs by one from its neighbour (Bauer et al, 1980). The fast migration of relaxed DNA relative to supercoiled DNA in agarose gels containing ethidium bromide has also been observed by Keller (1975b).

The DNA topoisomerase activity isolated from cdc7.4 cells showed optimal activity under conditions which differed from those reported for the same enzyme by Durnford and Champoux (1978) using a nitrocellulose filter binding assay. The assay used here is clearly superior, since it allows unequivocal interpretation of the extent of the reaction by visualization of the products.

Confirmation that the DNA topoisomerase could remove both positive as well as negative superhelical turns was obtained by performing reactions in the presence of varying concentrations (0, 1.5, 5.0 and 10.0 μg/ml) of ethidium bromide. High concentrations of dye cause relaxation of negative superhelical turns and the introduction of positive turns dependent on the amount of bound dye (Gale et al, 1972; Keller, 1975a; Champoux and Dulbecco, 1972). After reaction of enzyme and DNA as in Figures 68-70, enzyme and ethidium bromide were removed by extraction with phenol and then chloroform, the resulting DNA species were run on a 0.8% agarose gel (not containing ethidium bromide). Figure 71 shows that the DNA species which had been exposed to DNA topoisomerase and high ethidium bromide concentrations (5 and 10 μg/ml) migrated faster than DNA carrying the normal number of supercoils, due to the increased negative supercoiling following removal of ethidium bromide (Bauer et al, 1980). By defining a unit of DNA topoisomerase activity as an amount causing complete relaxation of 1nmole pJDB219 DNA in 10 minutes at 38°C, then the specific activity of fraction 31

(Figure 69) was 2950 units/mg protein.

6.3.4.7. Effect of DNA topoisomerase on *in vitro* DNA synthesis.

Benbow *et al* (1978) reported that DNA synthesis could be observed using ØX174 RF1 DNA as template in an *in vitro* system consisting of DNA polymerase α, RNA polymerase II and DNA topoisomerase from Xenopus laevis oocytes. The corresponding enzymes from yeast were assayed for activity on pJDB219 DNA, with a 4 hour incubation time at 30°C as used by Benbow *et al*, (1978). Under these conditions, yeast DNA polymerase I alone caused some incorporation of ^{3}H-dTMP into acid-precipitable radioactivity (data not shown). However, calf thymus DNA polymerase A2 showed no activity. The interpretation of this result, and the observations of Benbow *et al* (1978) comes from the observation that the DNA polymerase I and RNA polymerase II preparations possessed endonuclease activity (6.3.4.2) when assayed for an extended period (i.e. 4 hours). Thus the DNA synthesis stimulation seen by Benbow *et al* (1978) may have been due to a contaminating endonuclease activity in one of the enzyme preparations.

6.4. Conclusion.

This chapter has described attempts to observe initiation of DNA synthesis in a yeast *in vitro* system. Methods using complementation in whole cells necessitated the use of synchronised arrested cells. This caused problems in the interpretation of results, since cdc7.4 cells and spheroplasts, and also spheroplasts of wild-type A364A cells were found to be unstable, and therefore it proved difficult to distinguish complementation from stabilisation. A better and more direct approach was the attempt to reconstruct an *in vitro* DNA synthesising system from purified components. Thus far, meaningful initiation of DNA synthesis has not been observed using a supercoiled plasmid template. But there was evidence to suggest that the purified components were functional in these assays, and that the failure to synthesise DNA was due to the absence of an essential initiation factor(s), which may be the cdc7 gene product.

CHAPTER SEVEN

Summary and General Conclusion

CHAPTER 7.

Summary and General Conclusion.

Many approaches have been adopted in these studies in an attempt to discover the nature of the defect in cdc7.4. At this point it is useful to emphasize some of the difficulties which were encountered, which will bear on future studies. The cdc strains themselves, whilst entirely suitable for the genetic studies performed by Hartwell (review, 1978), have been found to possess characteristics unfavourable to the biochemical analyses performed here. The necessary restrictive temperature for cdc7.4, 38°C, lies at the limit of the growth capability of A364A, its wild-type parent, which can grow and synthesise DNA for only 2 generations under these conditions (3.3.1.1), after which time growth is clearly affected (5.3.1). Another wild-type strain, S2072D, is much more competent at growth at 38°C than A364A, but even this strain shows reduced overall growth at 38°C compared to 23°C (4.3.2.1). Further, cdc7.4 displays poor viability after more than 2 hours at 38°C (3.3.1.1.). This is a particular problem in studies in vivo, where an observed effect may be due to cell death, and not a direct consequence of the cdc7.4 mutation. The studies performed on spheroplasts as a prelude to the complementation assays using liposomes, confirmed the strain dependent nature of cell stability with respect to temperature. Spheroplasts prepared from wild-type A364A cells were much less competent at DNA synthesis at 38°C than similarly prepared cdc7.4 spheroplasts. The initiation phenotype of cdc7.4 necessitated that the conditions used in in vitro and in vivo studies were such that the cells or extracts were in the temperature blocked condition prior to use. This would not be the case for a DNA elongation mutant. These handling problems were the major barriers to the success of two complementation systems using

cdc7.4 spheroplasts.

Further problems have been manifest from the use of the cdc mutant collection. cdc7.4 (H201.14.4) was found to contain at least three separate mutations, namely, the defect in the initiation of DNA synthesis, a further ts lesion designated ts2 and an anomalous protein band at 72K MW on SDS-polyacrylamide gels. It is conceivable that other, as yet hidden, defects may also be present, probably as a consequence of the original mutant isolation procedure using NNG (Hartwell, 1967). The multiplicity of mutations lead to difficulties in the analysis of SDS-polyacrylamide gel patterns. It must be emphasised that the bands seen on one dimensional polyacrylamide gels represent moderately prevalent proteins within the cell. That band pattern differences exist between strains implies that it may be difficult to detect a mutant protein using exclusively this technique. The extra resolving power of 2D-polyacrylamide gel electrophoresis was found to be of little value when applied to extracts from whole cells because of obvious differences in the electrophoretic patterns of extracts from alleles of cdc7 which were allegedly isogenic (4.3.2.5). However, it may prove to be much more successful for the study of nuclear proteins. A method was described which gave highly purified nuclei in good yield. Using this preparation, an anomalous band pattern was seen when cdc7.4 (DE200.1.3) nuclei labelled at 38°C were compared with similarly labelled A364A nuclei (4.3.2.5). The validity of this observation has not yet been corroborated by comparison with the original cdc7.4 strain, H201.14.4. Clearly, further analysis is essential now that a collection of outcrossed cdc7 strains exist (DE100 and DE200 series), as well as the other alleles of cdc7 from the Hartwell collection. The difficulty in preparing nuclei from cells which have been incubated at 38°C (3.3.5.3), means that sample preparations can vary markedly, so a thorough investigation of this observation is necessary.

An observation of prime importance made in these studies, was that the replication of nuclear and 2 μm plasmid DNA was under the control of the CDC4, 7 and 28 gene products, and that mitochondrial DNA synthesis was not (3.3.3.2; 5.3.4.1). This meant that the yeast 2 μm plasmid could, in principle, be used to construct an *in vitro* system to study events during the initiation of nuclear DNA replication. An improved method for the preparation of 2 μm DNA in quantity was therefore derived. However this was eventually superseded when a recombinant plasmid, pJDB219, containing the entire yeast 2 μm plasmid genome (Beggs, 1978) became available.

Several enzymes which could possibly play a role in the initiation of replication were shown not to be thermosensitive in cdc7.4, namely RNA polymerases I, II and III, DNA polymerases I and II, DNA-dependent ATPase activity, SAM synthetase, DNA topoisomerase (nicking-closing enzyme) and an endonuclease which probably corresponds to endonuclease ∝ (Bryant and Haynes, 1978). However, a note of caution must be sounded, since the assays would probably not detect defects in regulatory subunits, or the *in vivo* functions may differ from those studied *in vitro*, as has been suggested for the DNA topoisomerase activity associated with the products of T4 genes 39, 52 and 60 (Liu *et al*, 1979; 1980).

Yeast DNA polymerase I, RNA polymerases I, II and III, a single-stranded DNA binding protein and DNA topoisomerase were partially purified. Some of these proteins have not been extensively studied previously. The single-stranded DNA binding protein, which may correspond to a protein of the same size (37K MW) isolated from a crude preparation of yeast RNA polymerase (Chang, L.M.S. *et al*, 1978) was shown to stimulate DNA replication by yeast DNA polymerase I and calf thymus DNA polymerase A2 on a synthetic template (6.3.4.3). This is an unusual feature, since other ssDBP's seem to stimulate only their homologous DNA polymerases (review Champoux, 1978). This protein is worthy of further study. The

DNA topoisomerase was purified essentially free of nuclease activity after only one chromatographic step. The fraction obtained was assayed for its activity on supercoiled DNA using agarose gel electrophoresis, which showed that the reaction conditions and assay described in a previous report (Durnford and Champoux, 1978) were unsatisfactory. This work is also the first time that the lack of thermosensitivity of the enzyme from cdc7.4 has been proved directly.

Attempts were made to observe initiation of DNA synthesis on supercoiled pJDB219 DNA using these partially purified proteins. This was not successful, but the components were shown to function individually in alternative assays, suggesting that the apparent failure was probably due to the absence of an essential function. The utilisation of single-stranded DNA by DNA polymerase I was shown to be enhanced by the addition of RNA polymerase I, presumably through the provision of ribonucleotide primers produced by the RNA polymerase (6.3.4.5). RNA polymerase I did not transcribe supercoiled DNA, but RNA polymerases II and II were shown to use different promoters on the pJDB219 plasmid. A problem with this type of <u>in vitro</u> assay is that initiation must be detected by subsequent DNA synthesis. Since the template is a supercoiled, double stranded DNA, the topological problems in replication are considerable, which means that activities capable of moving the replication fork must be present. Thus it might now be necessary to purify proteins with DNA-dependent ATPase activities, analogous to the rep protein (Yarranton and Gefter, 1979) and helicases I and II (Kuhn <u>et al</u>, 1979), of <u>E.coli</u>, and similar activities from higher eukaryotes, e.g. calf thymus (Assairi and Johnston, 1979), which have been implicated in this role. As yet, a similar protein has not been purified from <u>S.cerevisiae</u>.

Since relaxed plasmid DNA can now be obtained easily using the DNA topoisomerase, it is now possible to search for enzymes from <u>S.cerevisiae</u>, capable of the introduction of supercoils. This is of

particular relevance following the work of Liu et al, (1979, 1980) on the ATP-dependent DNA topoisomerase II required for the initiation of T4 DNA replication. There is evidence to suggest that E.coli DNA gyrase is required for the initiation of DNA replication (Filutowicz, 1980). Previously it had been believed that such activities were not necessary in eukaryotes, since the observed supercoiling of isolated SV40 chromosomes could be entirely accounted for by their interaction with histones (Champoux, 1978). However, the discovery of an activity in Xenopus laevis which can introduce supercoils into relaxed DNA (Tocchini-Valentini et al, 1978) questions this generalisation.

A further point concerning the methodology of future in vitro assays is that attempts to isolate a thermosensitive DNA initiation enzyme from extracts of cdc7.4 may be doomed to failure due to very low amounts of activity in the extract. This has been found to be the case for thymidylate synthetase from cdc21 (F. Z. Watts, unpublished observations; Bisson and Thorner, 1977) which had less than 0.2% of wild-type activity in vitro in extracts prepared from cells grown at 23°C. The dTMP labelling of DNA described in this work suggests that there is appreciable thymidylate synthetase activity in vivo at 23°C, indicating that the apparent lack of activity must be caused by enzyme instability during preparation of cell extracts. Thus it may be necessary to fractionate wild-type extracts to find activities which cause initiation, and then attempt to purify the similar protein from cdc7.4 to demonstrate its temperature-sensitivity.

The cdc7.4 gene product may not necessarily be a protein. Whilst this work has been in progress, evidence has appeared suggesting that a specific initiator RNA may be involved in the priming of ColE1 replication (Conrad and Campbell, 1979). Also, temperature-sensitive RNA species have been discovered (Belin et al, 1979). This idea must be borne in mind in further work on the in vitro assay system.

Another approach has been used in this laboratory to determine the nature of the cdc gene products. Work is in hand to clone the wild-type genes using hybrid vectors such as pJDB219. Ultimately, it is hoped to transcribe and translate the transforming DNA. For cdc7, when this point is reached, the final demonstration of the activity of the product will be the stimulation of DNA synthesis in an in vitro assay system.

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APPENDIX

Part of this work has been published in the following papers:

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